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Subcellular Localization Dynamics of Thyroid Hormone Receptor and Mediator Complex Subunit 1

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COMPLIANCE PAGE

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ABSTRACT

Intracellular trafficking of transcription factors is an essential cellular function that has implications in regulating gene expression. For thyroid hormone receptor (TR), nuclear localization is fundamental to its function of mediating gene expression in response to thyroid hormone (T3). Yet, we've previously shown that TR contains both nuclear localization signals and nuclear export signals, and shuttles rapidly between the nucleus and cytosol. Mislocalization of TR, and loss of transcriptional control, may lead to negative consequences for growth, development, and metabolism. Here, we explore factors that enhance nuclear retention of TR. Emerging studies suggest that Mediator complex subunit 1 (MED1 or TRAP220), a TR-interacting protein, may modulate nuclear retention of TR. To investigate this possibility, nucleocytoplasmic distribution and mobility of mCherry-tagged TR subtypes, TRα1, TRβ1, and the oncoprotein v-ErbA, were assessed in response to MED1 overexpression in HeLa cells. TRα1, which has a predominantly nuclear distribution at steady state showed no change in distribution pattern or intranuclear mobility when co-transfected with MED1. In contrast, overexpression of MED1 caused increased nuclear localization of TRβ1 and v-ErbA, subtypes with cytosolic populations at steady state, as well as a decrease in intranuclear mobility of TRβ1. Interestingly, in the presence of T3, which is known to induce phosphorylation of MED1, there was a decrease in both nuclear mobility and nuclear retention of TR subtypes. Using TRAP220$^{-/-}$ and TRAP220$^{+/+}$ mouse embryonic fibroblasts (MEF), TR localization in the absence of MED1 was subsequently analyzed. Compared to TRAP220$^{+/+}$ cells, TRα1 and TRβ1 showed an increase in cytosolic localization when expressed in TRAP220$^{-/-}$ MEFs. Taken together, our data provide evidence for MED1 promoting the nuclear retention of TRα1, TRβ1, and v-ErbA. Whether increased nuclear retention correlates with increased TR gene transactivation requires further analysis. Collectively, our findings implicate MED1 as a potential target in the pathogenesis of diseases that are linked to TR mislocalization and dysfunction.

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CHAPTER 1: GENERAL INTRODUCTION

The nuclear receptor (NR) superfamily is well-characterized for its vital role in regulating gene expression. The thyroid hormone receptor (TR), an important member of the NR superfamily, was discovered in the early 1970s, when its role in binding triiodothyronine (T3) was experimentally determined (Oppenheimer et al., 1972). Since its discovery, a tremendous amount of TR-related research has described its various functions and properties. TR functions primarily by binding to thyroid-hormone response elements (TREs) in target genes and either activating or repressing TR-regulated genes in response to T3. However, it has been shown that TR does not always remain in the nucleus and instead can translocate between the nucleus and the cytoplasm (Bunn et al., 2001). A significant shift in the balance of TR shuttling towards the cytoplasm, therefore, would make it impossible for TR to contact DNA and regulate gene expression. This conclusion led to our hypothesis that TR mislocalization is a contributing factor to disease pathogenesis (Bonamy et al., 2005; Bonamy and Allison, 2006), including the TRβ-centric resistance to thyroid hormone (RTH) and several types of cancer that result from failed regulation in the hypothalamic-pituitary-thyroid (HPT) axis (Brent, 2012; Cheng et al., 2010). Recently, the Allison lab has characterized the import and export mechanisms controlling TR's shuttling activity (Grespin et al., 2008; Mavinakere et al., 2012; Roggero et al., 2016; Subramanian et al., 2015). Yet, mechanisms for controlling TR nuclear retention are yet to be determined.

THYROID HORMONE

Circulation of thyroid hormone is initiated through a complex pathway spanning the HPT axis (Fig. 1). The system begins with a hypothalamic protein, thyrotropin releasing hormone (TRH), which is transported to and binds with its respective receptors in the pituitary gland (Yen, 2001). Here, TRH triggers the biogenesis of thyroid stimulating hormone (TSH) (Harris et al., 1978). TSH controls regulation of thyroid hormone synthesis through several pathways. In brief, they involve the secretion of TSH from the pituitary gland which leads to the interaction of TSH with TSH receptors (TSHR) in the thyroid (Yen, 2001). Activated TSHR prompts the expression of various proteins involved in synthesizing T3 and the more physiologically abundant 3,5,3′,5′-tetraiodo-L-thyronine, or thyroxine (T4). The concentration of thyroid hormone is regulated by thyroid hormone itself, which exerts negative regulation on TSH and TRH after a threshold is reached.

Of the total thyroid hormone produced, T4 is vastly more abundant than T3. Yet, T3 is the biologically active form of thyroid hormone that interacts with TR. While T4 is known for its longer half-life, its conversion to T3 is catalyzed by deiodinases. Most of the T3 circulating the body is bound to various molecules. It is estimated that only 0.3% of T3 is unbound and enters cells through facilitated transport mechanisms (Yen, 2001). Some of the transporters facilitating T3 cell entry include the monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), and various organic anion-

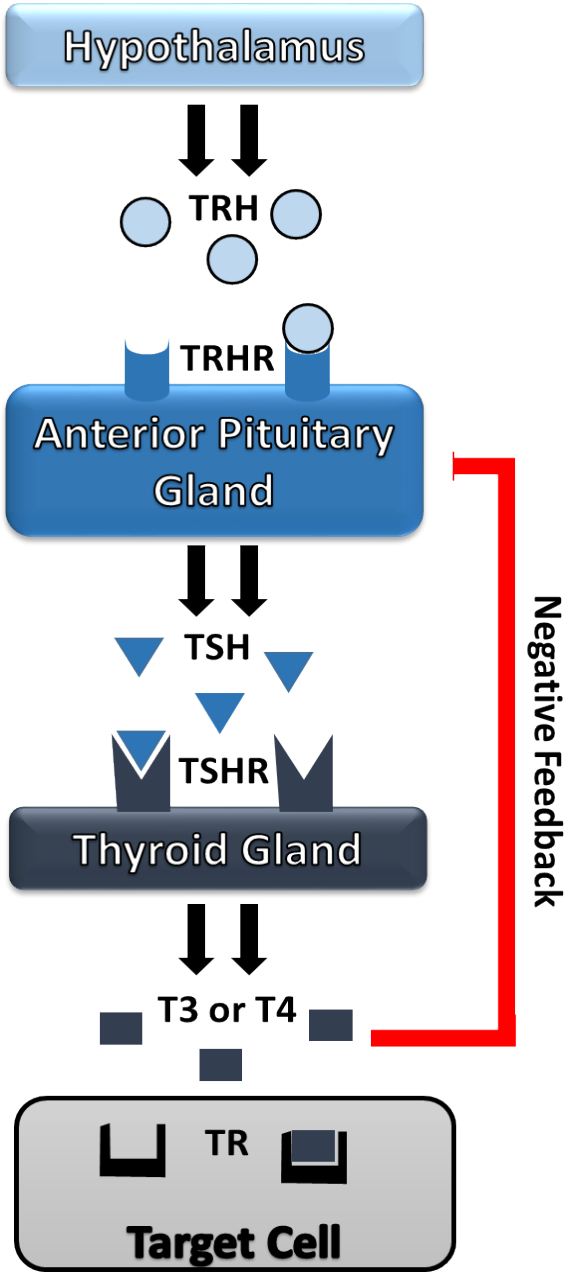

Figure 1. Thyroid hormone production pathway (A) Thyrotropin releasing hormone (TRH) is secreted from the hypothalamus. TRH binds to its receptors at the anterior pituitary gland. This leads to activation and release of thyroid-stimulating hormone (TSH). TSH binds to its receptors at the thyroid gland and thyroid hormone (T3/T4) is released. Thyroid hormone in its active form, T3, will interact with target tissue by binding to thyroid hormone receptor (TR). After sufficient T3 has been synthesized, it will exert negative feedback to prevent overproduction.

transporting polypeptides (OATP) (Visser et al., 2011). Once inside the cell, T3 can interact with TR and induce a cellular response.

THYROID HORMONE RECEPTORS

Genes and Distribution

TR is encoded by two separate genes in the human genome: *THRA* and *THRB*. Chromosome 17 contains *THRA* while *THRB* is positioned within chromosome 3 (Spurr et al., 1984; Weinberger et al., 1986). Numerous subtypes or isoforms of TR exist endogenously and in specific mammalian and viral systems. Expression of the *THRA* gene gives rise to TRα1 and, through alternative splicing, two other major isoforms: TRα2 and TRα3. Other isoforms also have been discovered by characterizing internal gene promoters (Brent, 2012; Cheng et al., 2010). Of the TRα isoforms, only TRα1 possesses the T3-binding site, due to the deletion of the site from the other isoforms during alternative splicing (Cheng et al., 2010; Mitsuhashi et al., 1988). Comparable to TRα, TRβ has three major isoforms: TRβ1, TRβ2, and TRβ3. The mechanism for producing these three separate isoforms for TRβ has been shown to arise from expression of the gene at three distinct promoter sites (Williams, 2000; Wood et al., 1994). All three isoforms of TRβ are capable of binding T3, however, the abundance of TRβ1 is greater and it has a wider distribution in mammalian systems than the other isoforms (Brent, 2012). Taking into account

the T3-binding ability of TRα1 and the historic scope of the Allison lab, the primary focus of this thesis is on TRα1 and TRβ1.

TRα1 and TRβ1 both exist universally in human tissues, but each is most abundant predominantly in specific and differing regions. TRα1 is found mainly in brain, heart, and skeletal muscle. TRβ1 is detected in kidneys, liver, brain, heart, and thyroid (Cheng et al., 2010). There is significant variability in the distribution and abundance of each isoform within different regions of the brain itself, as well. Additionally, TRα1 and TRβ1 are shown to be expressed at differing concentrations at various points in mammalian development (Bradley et al., 1992).

Protein Domains and Structure

Both TRα1 and TRβ1 share similarities in protein domain composition: an N-terminal A/B domain, a DNA-binding domain (DBD), hinge domain, and a ligand-binding domain (DBD) (Fig. 2). The LBD, as expected, interacts with thyroid hormone. Additionally, the LBD binds with a broad list of coactivators and corepressors. These coregulatory interactions can be directly involved in transcriptional regulation but also constitute a substantial list of non-transcriptional roles (Lonard and O'Malley, 2007). Similarly, the A/B domain is also commonly involved in transcriptional regulation. The DBD comprises the region of TR that interacts with thyroid hormone response elements (TRE). In terms of amino acid composition, TRα1 and TRβ1 differ primarily in the A/B domain, due to TRβ1 containing a relatively higher number of residues than

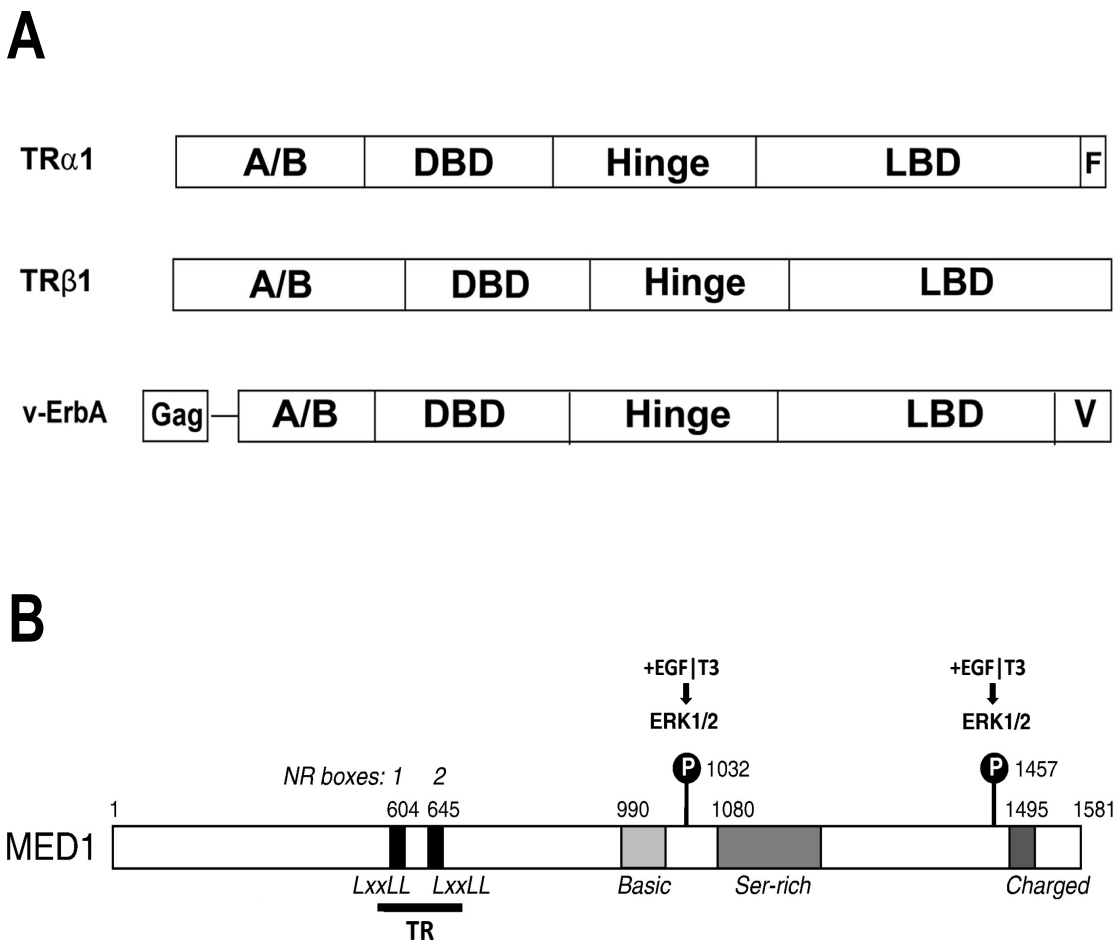

Figure 2. Protein structure of TRα1, TRβ1, v-ErbA and MED1 (A) Domain structures of TRα1, TRβ1, and v-ErbA (adapted from Mavinakere et al., 2012). The N-terminal A/B domain of TRβ1 has more amino acids than that of TRα1. v-ErbA has a viral Gag sequence N-terminal to the A/B domain. Besides several mutations in v-ErbA, the domain organization is conserved between the three. **(B)** Regions of MED1 identified for interaction with TR (adapted from Fondell, 2013). LxxLL motifs at 604 and 645 identify the point of contact between TRα1 and MED1. Thr-1032 and Thr-1457 are phosphorylated by MAPK-ERK when stimulated by EGF and/or T3.

TRα1 (Brent, 2012; Cheng et al., 2010). The A/B domain contains the activation function 1 (AF-1) region often implicated in transcriptional regulation (Barettino et al., 1994)

The LBD of both TRα1 and TRβ1 is highly conserved between the isoforms, as well as many NRs. A hydrophobic pocket is formed from the chiefly helical structure of the LBD, where T3 binds with high affinity to a conserved motif in helix 12 (Gronemeyer and Moras, 1998). Within the binding pocket, T4 has an acutely lower binding affinity than T3, which is thought to be due to single methionine and histidine residues (Wagner et al. 1995). The result leaves the TR-T4 interaction as sterically unfavorable. Of the 12 helices comprising the LBD, helix 12 has distinguished importance for all NRs due to its roles of repositioning the ligand and promoting altered interactions with coregulatory proteins (Gronemeyer and Moras, 1998). TR's helix 12 is contained within the highly conserved region of all NR LBD's designated the activation function 2 (AF-2) (Gronemeyer and Moras, 1998; Wagner et al. 1995). Akin to the AF-1 region, AF-2 binds to numerous coregulatory proteins (Barettino et al., 1994), including MED1/Trap220, the coactivator protein of interest in this thesis, and is therefore a significant component of the LBD moving forward.

v-ErbA, an oncogenic homolog of TR

In addition to TRα and TRβ isoforms, v-ErbA, a highly mutated form of TR has also been characterized and studied. v-ErbA is encoded by an oncogene carried by the avian erythroblastosis virus (AEV) (Beug and Hayman, 1992; Zenke et

al., 1990). As described in later sections, the resulting oncoprotein possesses several mutations which alter its nucleocytoplasmic transport compared to TR subtypes. The protein also has a C-terminal viral Gag sequence clearly not present in wildtype TR (Fig. 2). Mechanistically, AEV induces an overexpression of both v-ErbA and an oncogenic form of epidermal growth factor receptor (EGFR or HER1), v-ErbB (Beug and Hayman, 1992). Together, the overproduction of the oncoproteins leads to rapid and commonly fatal erythroleukemia in chickens (Beug et al., 1996). v-ErbA contributes to the oncogenesis in part by constitutively repressing transcription of genes crucial for proper erythrocyte functioning and differentiation (Ciana et al., 1998; Zenke et al., 1990).

v-ErbA also lacks the ability to bind to T3 (Sap et al., 1986). However, its primary association with AEV-induced tumorigenesis is amplified by v-ErbA's dominant-negative activity against TR. In one study, v-ErbA was shown to bind to TREs and effectively block TR from binding (Subauste and Koenig, 1998). As discussed below, TR must bind a TRE to regulate T3-responsive genes. Blocking the availability of response elements will consequently cause major problems in gene regulation. The oncoprotein can also exert dominant negative activity by binding to TR and forming a nonfunctional heterodimer that neutralizes the interactions required for TR gene regulation (Samuels and Selmi, 1991). Similarly, v-ErbA can interact with retinoid X receptor (RXR), a protein TR commonly forms heterodimers with, and block TR-RXR dimerization (Bonamy et al., 2005; Samuels and Selmi, 1991).

There is also evidence for v-ErbA altering TR's subcellular localization. Studies have shown that v-ErbA displays a predominantly cytoplasmic subcellular distribution at steady state (Bunn et al., 2001). Along with its mechanism for dimerizing with TR, the oncoprotein expands on this by sequestering a portion of the wild-type receptor to the cytoplasm and altering the export pathway of TR in a concentration-dependent manner (Bonamy et al., 2005). A closer look showed that cytoplasmic localization leads to the development of v-ErbA-induced aggresomes, or distinct puncta resulting from accumulation of misfolded proteins. This formation of aggresomes is thought to further augment the dominant negative activity of v-ErbA toward TR (Bondzi et al., 2011). This type of mislocalization of nuclear proteins to cytoplasmic compartments is a key factor in oncogenesis in many different systems. Notably, p53, a tumor suppressor, and a mutant form of BRCA1 associated with breast cancer development, acquire oncogenic activity following cytoplasmic mislocalization and sequestration (Bonamy and Allison, 2006; Rodriguez et al., 2004; Strommel et al., 1999). Unraveling different strategies against v-ErbA's actions has proven fruitful over the years and a portion of the studies presented in this thesis offers additional insight.

NUCLEOCYTOPLASMIC SHUTTLING

Nuclear Pore Complex

Early efforts in delineating basic structure of the cell nucleus revealed the identity of nuclear pore complexes (NPCs) (Callan and Tomlin, 1950). Since their discovery, the bidirectional transport of macromolecules between the nucleus and cytosol has been studied extensively. NPCs are massive protein complexes embedded in nuclear membranes that can range upward of 125 MDa in size (Reichelt et al., 1990). Architecture of an NPC is composed of a family of proteins called nucleoporins. Collectively, the nucleoporins assemble into the general NPC structure consisting of a central ring with asymmetric arrangement of fibrils extending freely on the cytoplasmic side but arranged into a 'basket' structure within the nucleoplasm (Hoelz et al. 2011). Proteins smaller than ~40 kDa pass through the central channel of the complex by diffusion; however, larger proteins require a facilitated transport mechanism discussed ahead. Although the exact biophysical forces driving interplay between transport proteins and the central nucleoporins are debated, the importance of interacting with phenylalanine-glycine repeats (FG repeats) of the nucleoporins to gain passage through NPCs is well-accepted (Hoelz et al., 2011; Kabachinski and Schwartz, 2015; Suntharalingam and Wente, 2003). To account for larger proteins passing through the relatively small diameter of the central ring, the functional diameter of NPCs is proposed to respond by dilating extensively (Pante and Kann, 2002).

Nuclear Localization and Export Sequences

Localization of proteins to the nucleus is dictated by the presence of either one or more nuclear localization signal (NLS) motifs. Classical monopartite and bipartite NLS motifs have been widely characterized (McLane and Corbett, 2009). For example, the classical monopartite NLS of simian virus 40 (SV40) contains a stretch of amino acids rich in lysine (K) and arginine (R). Furthermore, the classical bipartite NLS, which contains a region of linker amino acids flanked by two stretches of basic residues, is exemplified by the nucleoplasmin protein in *Xenopus laevis* (Kalderon et al., 1984; Kalderon et al., 1984; McLane and Corbett, 2009). Additionally, a class of NLSs (PY-NLS), characterized by the standard basic residues bordering proline (P) and tyrosine (Y) residues, along with non-classical NLSs have also been well-characterized (Süel et al., 2008; Lee et al., 2006; McLane and Corbett, 2009).

Proteins and RNA in the nucleus that are bound for the cytoplasm instead require nuclear export signals (NESs). Motifs for NESs aren't as well-conserved as NLSs; however, a common motif rich in leucine (L) and other hydrophobic residues is often present in NRs (Pemberton and Paschal, 2005; Wen et al., 1995). This leucine-rich hydrophobic motif has been shown to interact with CRM1 (chromosomal maintenance 1), also known as XPO1 (exportin 1), in a well-characterized CRM1-dependent export pathway (Fukuda et al., 1997; Ossareh-Nazari et al., 1997). Additionally, the CRM1-dependent export

pathway can be inhibited by a cytotoxic metabolite of *Streptomyces*, leptomycin B (LMB) (Fornerod et al., 1997, Wolff et al., 1997).

General Transport Mechanism

The general mechanism for nuclear import of proteins initiates with the recognition of an NLS by a specific class of transport receptors, referred to as karyopherins. Karyopherins involved with nuclear import, or importins, bind to their substrate's NLS and facilitate entry into the nucleus (Feldherr et al., 1984). As previously noted, translocation from the cytoplasm to the nucleus occurs through interaction of the importin with the interior FG repeats of the NPC (Hoelz et al., 2011). In the nucleoplasm, RanGTP then binds allosterically to the importin causing a conformational change and leading to the release of the cargo protein within the nucleoplasm (Gorlich et al., 1996). Nuclear export instead involves karyopherins termed exportins which interact with NES motifs on cargo proteins. Interaction of the exportin-cargo complex with RanGTP is required for the complex to exit out through the NPC (Izaurralde et al.,1997). In the cytoplasm, the export complex encounters proteins that trigger GTP hydrolysis by Ran. The switch to RanGDP induces a conformational change in the structure of the exportin causing the cargo to be released freely into the cytoplasm (Pemberton and Paschal, 2005). This Ran-mediated process is strictly dependent on a gradient created from the high concentration of RanGTP in the nucleus relative to the cytoplasm (Izaurralde et al.,1997; Macara, 2001).

Proteins containing both an NLS and NES are capable of translocation into and out of the nucleus. However, control of localization can be regulated through various post-translational modifications and inhibitory macromolecules 'masking' localization sequences to promote or inhibit proteins in the nucleus at a given time (McLane and Corbett, 2009). Although TR function is primarily as a transcription factor, heterokaryon and FRAP assays have previously shown that TR shuttles between the nucleus and the cytoplasm (Bunn et al., 2001; Grespin et al., 2008). Therefore, characterization of the various mechanisms balancing nuclear import, export, and retention are essential toward understanding systems where TR shuttling is proposed to be dysfunctional; i.e., RTH and types of cancer. Ultimately, a comprehensive map of these systems will assist in developing treatments for thyroid hormone-related diseases.

TR Nuclear Import

Nuclear localization signals in TRα1, TRβ1, and v-ErbA have been previously addressed. The Hinge domain of TRα1, TRβ1, and v-ErbA contains a well conserved classical bipartite NLS (Mavinakere et al., 2012). However, prior investigations pointed toward the presence of an additional NLS in TRα1 based on observations that TRβ1 exhibited a greater cytoplasmic distribution relative to TRα1 (Baumann et al., 2001; Zhu et al., 1998). NLS-2, a monopartite NLS, was subsequently identified in the A/B domain of TRα1; notably, this NLS is not present in TRβ1 (Mavinakere et al., 2012). Interestingly, a naturally occurring single amino acid substitution in the conserved region of NLS-2

present in v-ErbA completely ablates the function of the NLS (Mavinakere et al., 2012). Therefore, only TRα1 contains an active monopartite NLS-2.

In addition to the localization sequences that have been identified, the importins involved in facilitating nuclear import have been identified. The classical model for nuclear import involves the formation of a heterodimeric complex between adaptor importin α and importin β1, where importin α binds the cargo protein NLS and importin β1 interacts with the FG repeats of the NPC (Görlich et al., 1995; Lange et al., 2007). Recently, it has been shown that NLS-1 in the Hinge region of TRα1 and TRβ1 interacts with the importin α1/β1 complex (Roggero et al., 2016) (Fig. 3). Along with the importin α1/β1 pathway, importin 7 was also found to interact with NLS-2 of TRα1. However, since TRβ1 and v-ErbA both lack NLS-2, these proteins only utilize the importin α1/β1 import pathway (Roggero et al., 2016). Aside from the interactions between the importins and their respective NLSs, it has been shown that the A/B domain, DBD, and hinge domain are all required for complete nuclear localization of TRα1 (Mavinakere et al., 2012).

TR Nuclear Export

TRα1 and TRβ1 have been shown to contain a completely conserved NES in helix 12 of the LBD (NES-H12); however, v-ErbA lacks this sequence in the same position (Mavinakere et al., 2012). Two separate monopartite NESs have been identified in helix 3 (NES-H3) and helix 6 (NES-H6) of the TRα1 LBD and, based on sequence homology, are predicted to be within TRβ1 and v-ErbA, as

well (Mavinakere et al., 2012). v-ErbA uniquely possesses a leucine-rich NES present in the viral Gag sequence fused to the N-terminal end of the protein (DeLong et al., 2004).

TR was previously reported to only follow CRM1-independent export pathways, similar to many steroid receptors, due to the insensitivity of TR export to LMB (Black et al., 2001; Bunn et al., 2001). However, recently it has been shown that export of TR is facilitated through both CRM1-dependent and independent pathways. In the CRM1-dependent pathway, TRα1 interacts with a complex of CRM1 and calreticulin (CRT) which facilitates rapid export (Grespin et al., 2008). Additionally, the nuclear export of v-ErbA is completely controlled by the same CRM1-dependent pathway involving its leucine-rich NES (DeLong et al., 2004). However, when treated with LMB, TRα1 was still capable of export to the cytoplasm, solidifying the need to characterize a CRM1-independent export pathway (Grespin et al., 2008). Recently, exportins 4, 5, and 7 have collectively been found to be involved with this expected CRM1-independent export pathway (see Fig. 3). Similarly, TRβ1 export is facilitated by exportins 5 and 7 (Subramanian et al., 2015). The exact binding interactions between exportins and corresponding NESs on TRα1 and TRβ1 is still yet to be characterized.

NUCLEAR RETENTION

Proteins native to the nucleus often rely on several regulatory mechanisms to control not only functionality, but also nuclear localization. A central focus of this

thesis is to unravel fundamental ways in which TR, specifically, is retained in the nucleus rather than being exported. However, pursuing compartment-specific localization first requires an understanding of the biophysical dynamics of intranuclear protein movement.

Protein movement is commonly depicted as nonrandom motion facilitated by cytoskeletal motors (Brangwynne et al., 2008). While relevant, this thesis research instead focuses on protein migration described by Brownian motion. Early studies of Brownian motion explained how directional movement of foreign molecules in a solvent is affected by random collisions with the molecules constituting the solvent itself (Einstein, 1905). Application of these kinetics led to an understanding of how nuclear proteins adopt diffusional movement through the nucleoplasm (Phair and Misteli, 2000). Contrary to predictions based on Einstein's equation for diffusion, it has been shown that viscosity does not significantly affect the diffusion in different compartments (Einstein, 1905; Lukacs et al., 200). Instead, characterizing movement of proteins like NRs requires greater emphasis on the concentration of the proteins in bound versus unbound states. Therefore, freely diffusing NRs are identified as being in a "mobile" state. On the other hand, NRs actively involved in gene regulation will be interacting with DNA in an "immobile" state. Although bound versus unbound is frequently decided by protein-protein interactions, Phair and Misteli (2000) described how affinity for distinct sub-compartments of the nucleus can also further affect the estimated mobility of proteins. Collectively, persuasive strategies for assessing changes in nuclear retention of proteins requires

incorporation of assays aimed at both identifying compartmental localization of a protein and measuring intranuclear mobility.

A common method for studying intranuclear mobility is fluorescence recovery after photobleaching (FRAP) using confocal microscopy. FRAP takes advantage of proteins that are fluorescently labeled, such as nuclear proteins tagged with green fluorescent protein (GFP). The resulting excitation and emission of GFP can be ablated by extensive photobleaching. To achieve this irreversible photobleaching, confocal lasers are often set to maximal power briefly during imaging (time ≤ 1s) across a small section of the nucleus. As proteins diffuse through the nucleoplasm post-bleach, the unbleached fluorescent-proteins will eventually mix into the bleached region over time, yielding a recovery curve. The resulting recovery curve can then reveal dynamic properties of the nuclear protein such as recovery rate, mobile fraction, immobile fraction, half-time, and an estimated rate of diffusion through the nucleus (Carrero et al., 2003). Each of these factors are affected by the balance between mobile and immobile states of proteins.

As previously mentioned, the mobility of NRs can be affected by localizing to different compartments within the nucleus (Phair and Misteli, 2000). Beyond this, the mobility of TR is affected by other factors including protein-protein interactions, ligand-binding, and DNA binding (Rentoumis et al., 1990, Yen, 2001). TR FRAP studies have previously shown that the mobility of TR is relatively unaffected by the presence or absence of T3 (Maruvada et al., 2003).

However, it is possible that TR's interaction with coregulators may have a greater effect on TR's nuclear retention. Baumann et al. (2000) demonstrated how the presence of nuclear receptor corepressor (NCoR) increases TR's nuclear retention by decreasing nucleocytoplasmic shuttling; however, mutations that were introduced to disrupt protein-protein interactions would have disrupted NESs as well, so these studies remain inconclusive. Therefore, the potential of other coregulators to influence TR's retention requires further analysis.

TR TRANSCRIPTIONAL REGULATION

The main functional implications of TR nucleocytoplasmic shuttling are centered around its ability to regulate the expression of genes in response to thyroid hormone. Many NRs are sequestered to the cytosol in the absence of ligand and upon ligand interaction will translocate to the nucleus where they interact with DNA response elements (Yamamote, 1985). For example, in the absence of ligand, the glucocorticoid receptor (GR) is present in the cytoplasm bound to a heat shock protein 90 (hsp90) chaperone complex. Then, ligand-binding triggers a conformational change which allows for interaction of the nuclear import machinery with GR's NLS to then translocate the complex into the nucleus (Vandevyver et al., 2012). In contrast to other NRs, TR can constitutively bind to a TRE both in the presence and absence of its ligand, T3 (Rentoumis et al., 1990). During its interaction with TREs, TR can bind as a

monomer, homodimer, or heterodimer with other NRs. The retinoid X receptor (RXR) is commonly found to dimerize with TR (Xiao-kun et al., 1992). In the absence of ligand, TR dimers associate with a wide range of transcriptional corepressors. On the contrary, if T3 is present, TR undergoes conformational changes which release corepressor proteins and instead recruit coactivators. Part of this control can be attributed to the many negative and positive TREs discovered upstream and in untranslated regions of T3-regulated genes (Carr and Wong, 1994; Kim et al., 1992). In conjunction with the differing activity of response elements, a wide range of proteins have been characterized as coregulators that complement the regulatory functions of TR.

A commonly cited corepressor associated with TR is NCoR1 and its variants (Brent, 2012; Yen, 2001). NCoR1 has been shown to assist TR in repressing transcription of target genes (Hörlein et al, 1995). A similar protein, NCoR2 (also known as SMRT), was shown to have a nearly identical role as NCoR1. Together TR's interactions with NCoR1 and NCoR2 show that not only is the LBD important for coregulatory interaction but the Hinge domain, as well (Yen, 2001). TR and NCoR1 have also been implicated in interacting with various basal transcription factors (Baniahmad et al., 1993; Hörlein et al, 1995; Yen, 2001). Furthermore, TR and its NCoR1-silencing complex have been shown to interfere with the assembly of the transcriptional pre-initiation complex (PIC) as a mechanism for gene suppression in the absence of T3 (Fondell et al., 1993). TR's negative regulation of genes also extends to chromatin remodeling. Its association with various histone deacetylases (HDACs), such

as HDAC1, solidifies an epigenetic mechanism to introduce chromatin compaction and further inhibit assembly of the PIC (Alland et al., 1997). While corepressors are of interest to the Allison lab, this thesis focuses on TR's interaction with a coactivator.

Not surprisingly, there is also an exceedingly broad list of coactivators found to interact with TR. Steroid receptor co-activator 1 (SRC-1) has gained unique notoriety with TR due to its role in provoking RTH characteristics when its binding site in TR is mutated (Weiss et al., 1999). Another coactivator, TRIP230, has been shown to drastically upregulate the expression of TR-dependent genes (Chang et al., 1997).

Specifically, this thesis research focuses on factors that promote TR's nuclear retention. The problem with studying coactivators such as SRC-1 and TRIP230 is that they localize to many different organelles within the cell (Anbalagan et al., 2012; Chen et al., 1999). To find more promising candidates for promoting nuclear retention, we instead focused on TR-associated coactivators, in particular those identified as the thyroid-hormone associated proteins (TRAPs) (Fondell et al., 1996). This family of activators now is known to include many of the subunits that make up the transcriptional Mediator complex (Fondell et al., 1996; Ito and Roeder, 2001).

MEDIATOR COMPLEX AND PRE-INITIATION COMPLEX

The transcriptional pre-initiation complex (PIC) includes a diverse range of proteins complexed together to facilitate transcription. Incorporated into this massive structure is RNA Polymerase II (Pol II), basal transcription factors, coregulatory proteins, and chromatin remodeling complexes (see Fig. 3). Among these general PIC components is the Mediator complex, a 1.2 MDa composite of 26-30 subunits that was originally isolated from yeast (Thompson et al., 1993). The various Mediator subunits are isolated into four distinct regions of the complex. These structural regions are referred to as the head, middle, tail, and cyclin-dependent kinase (CDK) region (Wang and Yin, 2014). However, complexes isolated from various cell types have shown that several subunits of Mediator are variably absent from fully functional complexes (Allen and Taatjes, 2015). Although this may cause some dysfunction, it rarely disrupts the integrity of Mediator assembling the PIC (Allen and Taatjes, 2015). The CDK region provides an example of subunit variability, as this region has its own control mechanism for associating with and dissociating from Mediator that can dictate Mediator's interaction with Pol II (Knuesel et al., 2009). The control of the CDK region is situation-specific, such that CDK8 may be bound and function appropriately, but simultaneous binding of CDK19 with CDK8 is a proposed mechanism for disease onset (Allen and Taatjes, 2015).

Mediator is most notable for being involved in recruiting various members of the PIC to eukaryotic gene promoters (Baek et al., 2006). This includes direct

interaction with TFIIB and Pol II (Baek et al., 2006). However, Mediator has been implicated in all phases of transcription, from chromatin remodeling to splicing of the primary transcript. Additionally, Mediator is involved in in post-translational processing and has distinct enzymatic properties in various subcellular compartments (Poss et al., 2013). By association, the individual subunits are known to be incredibly versatile in function within the scope of these transcription-related processes (Yin and Wang, 2014). The bottom line is that Mediator plays a critical role in cellular and organismal development.

MEDIATOR COMPLEX SUBUNIT 1—TRAP220/MED1

MED1, also known as TRAP220, PBP, and DRIP205, is a 220 kDa, 1,581 residue protein subunit of Mediator. To make Mediator subunits more comparable between researchers, a naming system was initially agreed upon, deeming this subunit officially TRAP220 in human systems (Bourbon et al., 2004). However, a more recent adaptation of this system lists the Mediator subunits as “MEDs” (Robinson et al., 2015). For this thesis, depending on context, MED1 and TRAP220 will be used interchangeably.

MED1 is structurally found within the middle and tail region of Mediator. Here, it most notably interacts with MED26, which together, form an internal bridge between the middle and tail clusters (Poss et al., 2013)**.** The variable recruitment of MED1 and MED26 are unique to other Mediator subunits in that they are commonly found to both be absent from the same endogenous

Mediator complexes (Zhang et al., 2005; Taatjes and Tjian, 2004). Studies using MED1/TRAP220$^{-/-}$ mice have demonstrated the importance of MED1 during embryonic development (Ito et al., 2000). TRAP220$^{-/-}$ mouse embryos had failed organ development and eventually died (Ito et al., 2000; Landles et al., 2003). Therefore, although Mediator can function in the absence of MED1 in adult tissues, MED1 is clearly essential for proper development.

MED1 protein domains are still poorly defined; however, some distinct regions are currently well-characterized for interacting with NRs (see Fig. 2) (Fondell, 2013). The most relevant here are two regions termed nuclear-receptor boxes (NR boxes) or the preferred receptor-binding domains 1 and 2 (RBD-1 and RBD-2) which serve as sites for MED1-NR interactions. RBD-1 and RBD-2 both contain the conserved leucine-rich, LxxLL motifs, commonly found in coregulatory proteins that interact with NRs via the AF-2 region of the LBD (Gronemeyer and Moras, 1998; Ren et al., 2000). This interaction therefore has MED1 bridging the interaction between various NRs to the Mediator complex (see Fig. 3). The list of NRs interacting with MED1 includes TR, GR, vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), estrogen receptor, and androgen receptor (Chen and Roeder, 2007; Kang et al., 2002; Ren et al., 2000; Wang et al., 2002). Furthermore, Ren et al. (2000), found that TR, VDR, and PPAR, all bind with higher affinity specifically to MED1's RBD-2, rather than to RBD-1, due to a greater number of basic residues flanking RBD-2 which promote electrostatic interactions.

Aside from the RBDs, two threonines at residues 1032 and 1457 serve as targeted sites of phosphorylation for mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) (Pandey et al., 2005). More recent reports show that MED1 is also phosphorylated at serine-672 by CHK2 kinase, a mechanism implicated in DNA damage (Kim et al., 2017). Phosphorylation of MED1 leads to critical changes in protein activity. One study showed the phosphorylated MED1 has a significantly increased half-life in cells compared to the dephosphorylated form (Pandey et al., 2005). Phosphorylated MED1 also has an increased and augmented association with the Mediator complex due to greater interaction with the MED7 subunit (Belakavadi et al., 2008). Based on MED1's variable association with Mediator endogenously, phosphorylation has been hypothesized as a crucial post-translational modification regulating the interaction MED1 has with NRs, including TR (Belakavadi et al., 2008; Fondell, 2013; Pandey et al., 2005).

TR-MED1 INTERACTION

Previously, the Mediator complex has been probed for interacting with TR subtypes. Interaction of Mediator with TRα1 has been pursued far more than interaction with TRβ1; however, Zhu et al. (1997) showed that a synthetically truncated form of MED1 interacts with TRβ1. Studies on TRα1 and Mediator show direct interaction between MED1 and TRα1 (Fondell et al., 1996). As for other NRs, the proteins interact by contact between the LxxLL motifs in RBDs

of MED1 and residues in the C-terminal portion of TR's LBD (Ren et al., 2000; Yuan et al. 1998). Binding of MED1 with TRα1 also occurs in a ligand-dependent manner, observed by comparing liganded to unliganded TRα1, which shows no association with MED1 (Fondell et al., 1996). As previously discussed, binding of TR to its ligand, T3, is required to initiate TR's role as a transcriptional activator on positive TREs. Therefore, these previous findings reveal MED1's role as a coregulator of TR-associated genes.

Research groups have further investigated the MED1-TR interaction and the extent to which it affects TR-dependent gene expression. Studies using TRAP220$^{-/-}$ mouse embryonic fibroblasts (MEFs) demonstrated this point by showing that activation of TR-associated genes was inhibited in the TRAP220$^{-/-}$ cell, and that transcriptional output could be rescued by expression of exogenous MED1 in the fibroblasts (Ito et al., 2000; Malik et al., 2004). MED1 co-regulation of TR-associated gene expression can also be enhanced by ERK phosphorylation of MED1 (see Fig. 2). Furthermore, MAPK-ERK has been shown to be activated by T3, with levels proportionate to a known ERK activator, epidermal growth factor (EGF) (Pandey et al., 2005). Thus, not only does the presence of T3 and MED1's phosphorylation by MAPK-ERK regulate the TR-MED1 interaction, but it can also dictate the degree of TR-regulated gene transactivation (Pandey et al., 2005; Belakavadi et al., 2008). While MED1-TRα1 binding has been explored for its effect on gene expression, its effect on TR intracellular localization has not been investigated.

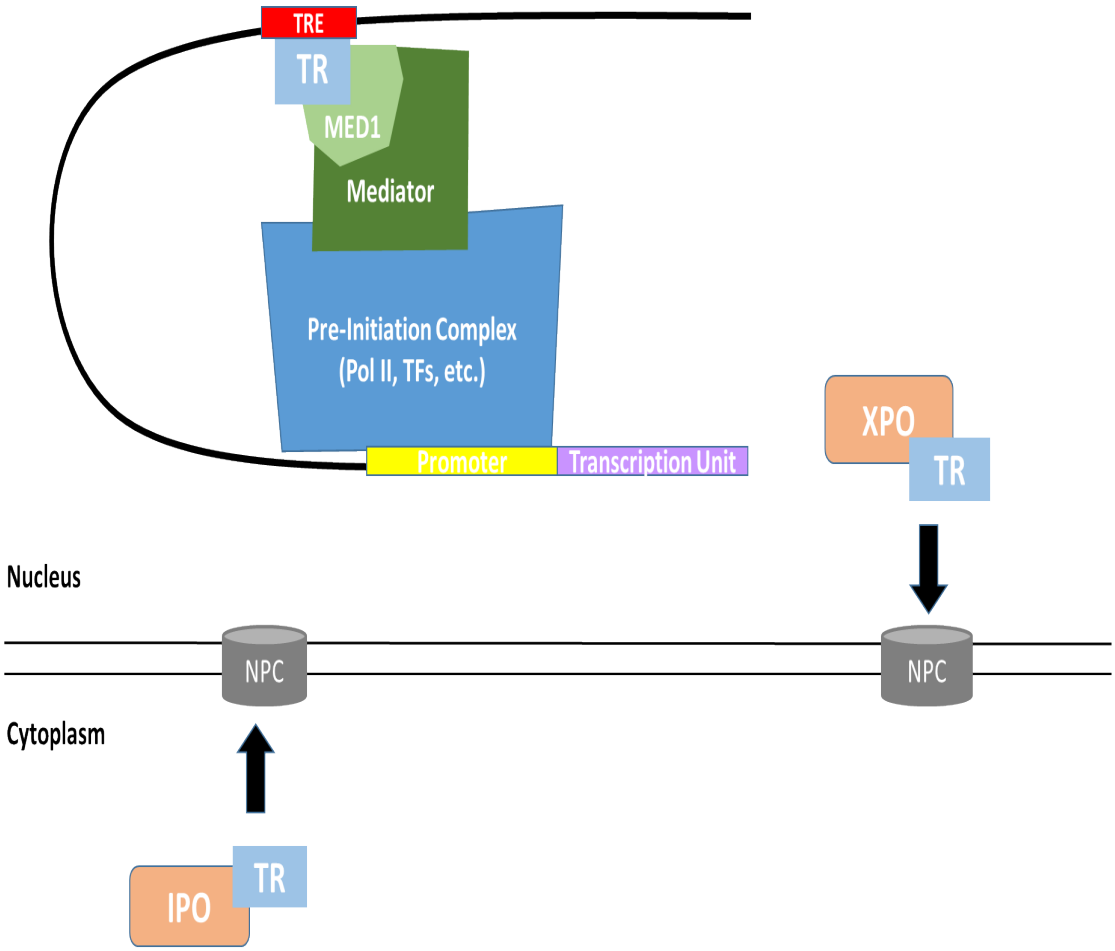

Figure 3. TR nuclear import, export, and interaction with MED1. Importin (IPO) α1, β1, and/or 7 facilitating import of TR through the NPC into the nucleus. In the nucleus, TR is bound to TRE while simultaneously interacting with MED1. MED1 is complexed within Mediator which is functioning as a component of the PIC. To shuttle back into the cytoplasm, TR binds with exportin (XPO) 4, 5, and/or 7 and the translocates back out through an NPC.

THESIS OBJECTIVE

Retention of TR within the nucleus is essential for TR-associated gene silencing or transactivation. Characterization of TR shuttling between the nucleus and the cytosol, along with identification of multiple import and export signals, has highlighted the need to elucidate how the balance between nuclear import, export, and retention is maintained. Further insight into the relationship between these mechanisms will provide a better understanding of how TR mislocalization may lead to disease (Bonamy et al., 2005; Bonamy and Allison, 2006; Mavinakere et al., 2012). TR's nuclear import and export systems have garnered considerable attention recently, which has revealed many components. The Allison lab has previously shown that T3 is one factor that promotes the nuclear retention of TR (Bonamy and Allison, 2006; Bunn et al., 2001); however, additional mechanisms for nuclear retention of TR are poorly defined.

The overall objective of this thesis research was to expand understanding of factors that play a role in TR nuclear retention. As discussed, great attention has been given to MED1's role as a coactivator for TR-regulated gene transcription. Therefore, MED1 provides a promising candidate for influencing TR's retention in the nucleus (see Fig. 3). This thesis research specifically focused on the effect of MED1 on TR's nucleocytoplasmic distribution and intranuclear mobility. These two characteristics were assessed when MED1 was overexpressed, knocked-out, or phosphorylated. Additionally, previous

studies on MED1 and TR have primarily dealt with TRα1, and some aspects of this earlier work also used synthetically truncated forms of MED1, particularly for analysis of phosphorylated MED1. Therefore, part of this thesis was also devoted to examining MED1's relationship with TRβ1 and the oncoprotein v-ErbA.

The following three specific aims were investigated in this thesis research:

1. Evaluate if overexpression of MED alters the nuclear retention of TR
2. Assess the impact that knockout of MED1 has on nuclear retention of TR
3. Elucidate how nuclear retention of TR is affected by MED1 phosphorylation by ERK.

CHAPTER 2: MANUSCRIPT DRAFT FROM THESIS RESEARCH

NUCLEAR RETENTION OF TRα1, TRβ1, AND v-ErbA IS DIFFERENTIALLY REGULATED BY MED1/TRAP220

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ABSTRACT

Intracellular trafficking of transcription factors is an essential cellular function that has implications in regulating gene expression. For thyroid hormone receptor (TR), nuclear localization is fundamental to its function of mediating gene expression in response to thyroid hormone (T3). Yet, we've previously shown that TR contains both nuclear localization signals and nuclear export signals, and shuttles rapidly between the nucleus and cytosol. Mislocalization of TR, and loss of transcriptional control, may lead to negative consequences for growth, development, and metabolism. Here, we explore factors that enhance nuclear retention of TR. Emerging studies suggest the likelihood that Mediator complex subunit 1 (MED1 or TRAP220), a TR-interacting protein, modulates nuclear retention of TR. To investigate this possibility, nucleocytoplasmic distribution and mobility of mCherry-tagged TR subtypes, TRα1, TRβ1, and the oncoprotein v-ErbA, was assessed in response to MED1 overexpression in HeLa cells. TRα1, which has a predominantly nuclear distribution at steady state showed no change in distribution pattern or intranuclear mobility when co-transfected with MED1. In contrast, overexpression of MED1 caused increased nuclear localization of TRβ1 and v-ErbA, subtypes with cytosolic populations at steady state, as well as a decrease in intranuclear mobility of TRβ1. Interestingly, in the presence of T3, which is known to induce phosphorylation of MED1, there was a decrease in both nuclear mobility and nuclear retention of TR subtypes. Using TRAP220-/- and TRAP220+/+ mouse embryonic fibroblasts (MEFs), TR localization in the

absence of MED1 was subsequently analyzed. Compared to TRAP220+/+ cells, TRα1 and TRβ1 showed an increase in cytosolic localization when expressed in TRAP220-/- MEFs. Taken together, our data provide evidence for MED1 promoting the nuclear retention of TRα1, TRβ1, and v-ErbA. Whether increased nuclear retention correlates with increased TR gene transactivation requires further analysis. Collectively, our findings implicate MED1 as a potential target in the pathogenesis of diseases that are linked to TR mislocalization and dysfunction.

INTRODUCTION

Thyroid hormone receptor (TR), a member of the (NR) superfamily, is expressed as two major subtypes, TRα1 and TRβ1. TR functions primarily as a transcription factor capable of both activating and inhibiting the expression of various genes in response to thyroid hormone (T_3). In contrast to many nuclear receptors that require interaction with ligand before translocating to the nucleus and interacting with DNA, TR is constitutively bound to its respective thyroid hormone response element (TRE), in the absence of ligand (Brent, 2012). Constitutive interaction with DNA implies that TR would have a predominantly nuclear localization. However, we previously demonstrated that TR exhibits shuttling activity between the nucleus and cytoplasm (Bunn et al., 2001). Mislocalization of TR to the cytoplasm has the potential to disrupt its role regulating gene expression. Studies using v-ErbA, an oncogenic homolog of TR

that localizes mainly to the cytoplasm, point to mislocalization as a possible mechanism for oncogenesis (Bonamy et al., 2005: Bonamy and Allison, 2006; Bondzi et al., 2011). Resistance to Thyroid Hormone (RTH), a disease which causes symptoms of both hypo- and hyperthyroidism, is also linked to several TR mutations, primarily with TRβ (Yen et al., 2001), and mislocalization may contribute to its development (Mavinakere et al., 2012). More recently, several different mutations have been found in TRα1 that lead to RTH (Schoenmakers et al., 2013). Therefore, unraveling how the balance of TR's nuclear import, retention, and export is controlled will provide further insight into TR-associated disease pathogenesis.

Recently, our understanding of TR's nuclear import and export pathways has expanded considerably. In addition to a well-conserved nuclear localization signal (NLS) in the Hinge domain of TRα1 and TRβ1, termed NLS-1, TRα1 has an additional NLS, NLS-2, in its A/B transactivation domain which enhances its localization to the nucleus relative to TRβ1 (Mavinakere et al., 2012). NLS-1 facilitates nuclear import through interaction with the importin α1/β1 karyopherin complex, while NLS-2 is associated with the importin 7 pathway (Roggero et al., 2016). In addition to import signals, multiple nuclear export signals (NESs) have been characterized, as well. Two distinct monopartite NESs were characterized within helix 3 and helix 6 of TRα1 and predicted to also be within TRβ1 and v-ErbA (Mavinakere et al., 2012). However, an additional NES is also found within helix 12 (NES-H12) of TRα1 and TRβ1's ligand binding domain (LBD), which is absent in v-ErbA (Mavinakere et al., 2012). Instead, v-ErbA has a unique NES

in its C-terminal viral Gag sequence (DeLong et al., 2004). Nuclear export occurs through both CRM1-dependent and CRM1-independent pathways. Different from TRα1 and TRβ1, v-ErbA is strongly exported by a CRM1-dependent pathway, resulting in greater cytoplasmic distribution relative to TRα1 and TRβ1 (Bunn et al., 2001; DeLong et al., 2004). CRM1-dependent TR export involves interaction with calreticulin and can be partially inhibited by Leptomycin B, a cytotoxin produced by *Streptomyces* (Fornerod et al., 1997, Grespin et al., 2008; Wolff et al., 1997). On the other hand, CRM1-independent export of TR involves several other exportins. Our investigation of this mechanism revealed that TR interacts with exportins 4, 5, and 7 (Subramanian et al., 2015). While recent discoveries have enhanced our understanding of nuclear import and export, characterization of factors promoting TR's retention to the nucleus remain unclear.

TR has been found to interact with various subunits of the Mediator complex. Specifically, TRα1 and TRβ1 directly bind with Mediator complex subunit 1 (MED1), also called TRAP220 (Fondell et al., 1996; Zhu et al., 1997). Receptor binding domains present in MED1 contain leucine-rich LxxLL motifs which interact with the activation function 2 (AF2) region of TR's LBD (Ren et al., 2000; Yuan et al. 1998). Phosphorylation of MED1 by MAPK-ERK at two threonine residues further promotes this interaction and can be stimulated by both epidermal growth factor (EGF) and T3 (Pandey et al., 2005). This phosphorylated state also activates TR's role as a coactivator for TR-regulated gene expression (Belakavadi et al., 2008). Conversely, TR's gene

transactivation activity has been shown to be ablated in MED1/TRAP220$^{-/-}$ mouse embryonic fibroblasts (MEFs), but can be reversed by adding MED1 back into the cells (Ito et al., 2000; Malik et al., 2004). Collectively, studies have established the significance of binding MED1 for proper TR-associated transcriptional regulation.

Here, we investigated how the presence or absence of MED1 affects nuclear retention of TRα1, TRβ1, and v-ErbA in transfected HeLa cells by using fluorescence recovery after photobleaching (FRAP) mobility assays and fluorescence-assisted nucleocytoplasmic scoring. Our data suggest that MED1 indeed plays a significant role in the nuclear retention of TRα1 and TRβ1. MED1 also promotes retention of v-ErbA; however, coimmunoprecipitation experiments need to be implemented to determine whether this is from directly binding with MED1 or by an alternative indirect mechanism. We also sought to assess the impact of MED1 phosphorylation by MAPK-ERK on TR's localization. We found that T3-stimulated phosphorylation of MED1 led to increased mobility of TRβ1 and decreased nuclear retention. Additionally, EGF-stimulated MED1 phosphorylation caused a reduction in the mobile fraction of TRα1. Collectively, our assays suggest that MED1 is a key factor in decreasing the intranuclear mobility of TR and thereby promoting its nuclear retention.

EXPERIMENTAL PROCEDURES

Plasmids – Plasmids were bacterially cultured and purified from competent *E. coli.* EGFP-C1 and pmCherry-C1 were obtained from Clontech Laboratories, Inc. Preparation of the mCherry-TRα1 and mCherry-v-ErbA expression vectors were performed by subcloning the corresponding coding regions from previously prepared GFP-TRα1 and GFP-v-ErbA (Bunn et al., 2001) into the pmCherry-C1 (Clontech) vector. mCherry-TRβ1 was similarly prepared (by V. Roggero) through subcloning the TRβ1 coding sequence from GFP-TRβ1 (Subramanian et al., 2015), encoding functional GFP-tagged human TRβ1, into the pmCherry-C1 vector. GFP-GST-GFP(G3)-Hinge, containing the TR NLS-1, expression plasmid was previously described (Mavinakere et al., 2012). GFPSpark-MED1 was obtained from Sino Biological Inc. and encodes human MED1, truncated from residues 547-1581. The expression plasmid for GFP-tagged full length human MED1 (residues 1-1581) was acquired from OriGene Technologies, Inc. pEGFP-GR expression plasmid was from Addgene (plasmid #47504; Alice Wong).

Mouse Embryonic Fibroblasts (MEFs) – Assays investigating the effects of MED1 knockout were conducted using Trap220$^{+/+}$ and Trap220$^{-/-}$ MEFs. The MEFs were a gift from the lab of Dr. Robert Roeder (Rockefeller University). Further details regarding both the preparation of the MEFs and generation of Trap220$^{+/+}$ and Trap220$^{-/-}$ mice can be found in Ito et al. (2000).

Cell Culture and Transfection - HeLa cells were cultured in Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), or 10% FBS-MEM, at 37˚C, 5% CO_2, and 98% humidity to 70 – 90% confluency. MEFs were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% Newborn Calf Serum (Gibco), or 10% NCS-DMEM. Cells were displaced with 0.25% trypsin (Gibco). 6-well culture dishes were seeded at a density of 2.5 x 10^5 per well on coverslips and incubated for ~24 h. Plates were then transfected or cotransfected with 1 µg of either mCherry-TRα1, mCherry-TRβ1, or mCherry-v-ErbA and 1 µg of either truncated or full-length GFP-MED1 expression plasmids. Lipofection for HeLa cells was facilitated by Lipofectamine 2000 (Invitrogen) in Opti-MEM 1 Reduced Serum Medium (Invitrogen). Lipofectamine 3000 (Invitrogen) in Opti-MEM 1 Reduced Serum Medium (Invitrogen) was used for transfecting MEFs. Both Lipofectamine 2000 and 3000 were used according to the manufacturer's protocol.

Quantitative Analysis of Nucleocytoplasmic Distribution – Eight hours post-transfection, transfection mixtures were replaced with the respective 10% FBS-MEM or 10% FCS-DMEM medium. Approximately 24-26 h post-transfection, cells were washed as previously described (Bunn et al., 2001) and fixed in 3.7% formaldehyde. Coverslips were then mounted with Fluoro-Gel II containing DAPI onto glass slides. Protein distribution was analyzed using a Nikon Plan Apo 40x/0.95 objective on a Nikon ECLIPSE TE 2000-E fluorescence microscope and fluorescence emittance was facilitated by the following filter sets: Nikon Ultraviolet Excitation via UV-2E/C filter for DAPI/nuclei visualization;

Blue Excitation via B-2E/C filter block for GFP/FITC visualization; and Red Excitation via T-2E/C filter for mCherry/TRITC. A CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ) allowed image capture and NIS-Elements AR software (Nikon) was used for analysis. Prior to analysis, slides were blinded by members of the lab to ensure scoring was performed without knowledge of treatment. Regions of interest (ROI) were positioned inside both the nucleus and cytoplasm of cells and fluorescence intensity was recorded for each. Three biological replicates with a minimum of 100 ROI-analyzed cells were recorded per transfection combination of TR subtypes with MED1. Relative nuclear to cytoplasmic (N/C) distribution and analysis was then calculated and normalized for corresponding biological replicates in Excel. Cotransfected ratios were normalized against the corresponding single transfection, where cotransfected TR would be normalized against the TR individually transfected under the same conditions. A ratio greater than a normalized value of 1 was interpreted as having a more nuclear distribution, while less than one indicated a greater distribution of protein in the cytoplasm. A student's T-test was used to calculate p-values and determine significance.

Confocal Fluorescence Recovery After Photobleaching (FRAP) – Eight hours post-transfection, transfection mixtures were replaced with the respective 10% FBS-MEM or 10% FCS-DMEM medium. Twenty three to twenty nine hours post-transfection, cells were washed with Dulbecco's phosphate buffered saline (D-PBS). Cells were then incubated in MEM-α medium containing 50 µg/mL cycloheximide, 50 units/mL penicillin, and 50 µg/mL streptomycin for the

duration of the assay. After the addition of MEM-α, plates were incubated in an OkoLab Incubation System (Warner Instruments, Inc., Hamden, CT) which maintained conditions of 37°C and 5% CO_2. To verify consistency of treatment results between the 6 h period, each treatment was assessed at the 23 h, 26 h, and 29 h intervals for the three biological replicates. A Nikon A1Rsi confocal microscope Ti-E-PFS (Nikon Inc., Melville, NY) with a 60x oil objective was utilized for all FRAP experiments. The 488-nm line of krypton-argon laser with a band-pass of 525/50 nm emission filter was used for GFP detection, while the 561-nm line with a band-pass emission filter was used for mCherry detection. A solid-state 405-nm line of laser with a band-pass 450/50 emission filter was used exclusively for photobleaching. The “Perfect Focus System” (PFS) was applied during the duration of the experiments. Both acquisition and photobleaching were coordinated within NIS-Elements AR (Nikon). Using the stimulation module of NIS-Elements, the total experimental time for the assay was ~35 s. This was divided into a 5 s “pre-bleach” acquisition phase at ~2-3% laser power, 1 s of photobleaching at 100% laser power, and a post-bleach acquisition phase, again, at 2-3% laser power. All image acquisition was conducted through resonant scanning. The poor signal to noise ratio associated with both resonant scanning and low laser power was corrected by line averaging. Data from three biological replicates of 20 nuclei from separate cells were recorded for each TR isoform, MED1 variant, and cotransfection combination between the two.

FRAP data were normalized using a modified strategy presented by Phair et al. (2003). Equation 1 shows full normalization of fluorescence, I_{full}, as a function of time, where $I_{double\ norm}$ represents the background-corrected, double normalization of fluorescence and $t_{postbleach}$ is the background-corrected value at the time point immediately after bleaching has terminated. Further description of the double normalization is outlined by Phair et al. (2003). Effectively, the data were normalized from 0 – 1, where 0 was the lowest relative intensity, directly after the bleaching phase, and 1 was the point of greatest post-bleach recovery.

$$I(t)_{full} = \frac{I(t)_{double\ norm} - I(t_{postbleach})_{double\ norm}}{1 - I(t_{postbleach})_{double\ norm}} \quad (1)$$

Normalized data were then used to calculate the rate of recovery for each treatment. The recovery rate, post-bleach fluorescence recovery over time to equilibrium, was used as the primary measurement to evaluate changes in mobility. The normalized data were also used to estimate a half-time/T_{half} value, or the time required for half of the total fluorescence to recover back into the bleached region. As our data were normalized to 1, we recorded the half-time as the time that elapsed between the end of the bleaching phase and the point at which 50% recovery occurred directly on the full-normalized recovery curve. Aside from T_{half}, we also calculated the mobile and immobile fractions to support our understanding of mobility dynamics. The mobile fraction is represented in Equation 2

$$F_m = \frac{I(t_{eq}) - I(t_{postbleach})}{I(t_{pre}) - I(t_{postbleach})} \quad (2)$$

where $F_{m,}$ is the mobile fraction, $I(t_{eq})$ is the full normalized intensity at which equilibrium on the recovery is reached, $I(t_{pre})$ is defined by the intensity pre-bleach, and $I(t_{postbleach})$ is the intensity at time (t), directly after bleaching. Subsequently, the immobile fraction F_i can be calculated using Equation 3

$$F_i = 1 - F_m \quad (3)$$

Induction of MED1 Phosphorylation – Eight hours post-transfection, transfection mixtures were replaced with 10% Charcoal Stripped FBS (Gibco) MEM, or CS-MEM, and/or either human epidermal growth factor, EGF (Gibco), or T3 (Sigma). T3 and EGF were supplemented at previously described concentrations of 100 nM and 100 ng/ml, respectively (Bunn et al., 2001; Pandey et al., 2005). Twenty four to twenty six hours post-transfection, samples were then prepared and analyzed using the appropriate methods described for either FRAP or N/C nucleocytoplasmic scoring.

Leptomycin B and Dexamethasone Treatment – FRAP assays performed on cells expressing mCherry-v-ErbA were supplemented with 5 ng/mL of Leptomycin B (Sigma) ~45 minutes prior to analysis. Cells transfected with GFP-tagged GR were treated with dexamethasone (Sigma) at a final concentration of 1 μM and incubated for ~30 minutes prior to analysis. Optimal minimum incubation times were determined separately using time lapse imaging. Directly after treatments were added, samples were transferred to the

confocal microscope setup (*Confocal Fluorescence Recovery After Photobleaching (FRAP))* and monitored for the accumulation of fluorescent protein in the nuclei of multiple cells. When fluorescent signal comparable to previously documented nuclear-localized protein samples, such as mCherry-TRα1 or GFP-MED1, was reached, the minimum incubation period was achieved.

Validation of MED1 Knockout and Phosphorylation

HeLa cells and MEFs were seeded at 8.0 x 10^5 cells per 100 mm dish. HeLa (phosphorylation assay) and MEF (knockout validation) lysates were prepared 48 h after cells were plated. Protein concentrations were measured using a Nano Drop (ND-1000 Spectrophotometer. 40 – 50 µg of protein were analyzed per lane. Western blot was performed as previously described (Subramanian et al., 2015). Antibodies were used with the following concentrations: anti-GAPDH (Santa Cruz Biotechnology Inc, Dallas, TX), 1:5000; anti-MED1 (Abcam), 1:500; anti-phosphothreonine (Abcam), 1:125; horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare Life Sciences), 1:25,000; HRP-sheep anti-mouse IgG (GE Healthcare Life Sciences), 1:25,000. Protein size was confirmed using Pre-Stained Kaleidoscope Protein Standards (Bio-Rad, Hercules, CA). X-ray films were quantified by scanning densitometry using NIH ImageJ software.

RESULTS

Overexpression of MED1 increases nuclear retention of TRβ1 and v-ErbA. Previous studies have determined that although TR localizes primarily to the nucleus, there is definitive shuttling between the nucleus and cytoplasm, as well (Bunn et al., 2001). The constitutive presence of TR in the nucleus points to regulatory factors that promote nuclear retention of TR. Here, we investigated the possibility that MED1, a transcriptional coactivator, plays a key role in TR nuclear retention.

To determine if overexpression of MED1 altered the localization of TRα1 and TRβ1, HeLa cells were transfected with either mCherry-TR or GFP-MED1 individually and compared to cells cotransfected with mCherry-TR and GFP-MED1. Each biological replicate contained a sample with mCherry-tagged TR, GFP-MED1, and one with TR and MED1 cotransfected. The single transfections were used as controls to normalize the fluorescence intensity of cotransfections for each replicate, and to calculate the comparative N/C ratio, to determine whether there were statistically significant differences in localization between treatments. As a negative control, we performed the same individual transfections and cotransfections, using a truncated form of MED1 (tMED1), which lacks the RBDs of wild type MED1 which interact with the LBD of TR. We predicted that overexpressing full-length MED1 would lead to a greater distribution of both TRα1 and TRβ1 in the nucleus, while overexpressing the truncated MED1 would have no effect on the distribution pattern of TR. As

predicted, when cotransfected with tMED1, there was no significant change in localization of TR (data not shown).

Consistent with previous studies (Zhu et al.,1998; Bunn et al., 2001; Roggero et al., 2016), TRα1 was localized almost entirely to the nucleus, with only a small population of cells (<10%) having a cytosolic population of TRα1 (Fig. 1), whereas TRβ1 showed a greater cytosolic distribution relative to TRα1 (Fig. 2). When TRα1 was cotransfected with MED1, we observed no significant change in TRα1's localization ($p=0.299$) (Fig. 1). However, cotransfection of MED1 and TRβ1 caused a significant shift toward a greater nuclear localization of TRβ1 ($p=0.004$) (Fig. 2), suggesting that MED1 promotes TR nuclear retention. Based on this shift towards greater nuclear retention of TRβ1 in the presence of MED1, it seems likely that nuclear retention of TRα1 was also increased. However, due to the already robust localization of TRα1 in the nucleus at steady state, even without overexpression of MED1, it was not possible to detect enhanced retention.

In our earlier reports, we have shown that in most cells v-ErbA, the oncogenic homolog of TR, has a predominantly cytoplasmic distribution (Bunn et al., 2001; Bonamy et al., 2005; DeLong et al., 2004). Individual transfection of mCherry-v-ErbA reaffirmed this finding (Fig. 3). In contrast, we found that overexpressing MED1 led to a more whole-cell distribution of v-ErbA; there was a significant increase in the relative N/C ratio ($p=0.001$) (Fig. 3), suggesting that MED1 also promotes nuclear retention of v-ErbA.

Cotransfecting MED1 with TR subtypes alters the nuclear distribution of MED1. Although our primary interest is with the subcellular localization of TR, we also analyzed the distribution pattern of MED1 alone and when cotransfected with TR subtypes. Previous reports confirmed the predicted nuclear localization of MED1 (Cui et al., 2012; Fondell, 2013; Pandey et al., 2005), and there is no evidence yet of rapid nucleocytoplasmic shuttling of MED1 (Allison lab unpublished observations). Therefore, we predicted our nucleocytoplasmic scoring method would show MED1 has near-absolute nuclear localization. Here, we verified the distinct nuclear localization of MED1 when transfected alone (see Figs. 1 – 3). Interestingly, nearly half of the cells transfected with only GFP-MED1 contained nuclei with several MED1-aggregates of either approximately equal size or far larger aggregates that encompassed up to half of the nucleus (see Fig. 3, “MED1 single” panel). Cotransfecting any of the TR variants with MED1 resolved these nuclear aggregates. Furthermore, we discovered that overexpressing MED1 with TRα1, TRβ1, or v-ErbA, simultaneously, resulted in a significant shift towards a more cytosolic localization of MED1 ($p<0.001$, $p<0.05$, and $p<0.001$, respectively). The physiological relevance of these observation remains to be determined.

Overexpression of MED1 alters the intranuclear mobility of TRβ1. The reported role of MED1 as a coactivator of TR-regulated genes (Belakavadi et al., 2008; Fondell, 2013; Pandey et al., 2005) led us to hypothesize that overexpressing MED1 would increase TR’s residence time bound to DNA. As a result, we predicted that overexpression of MED1 would lead to a decrease in TR

intranuclear mobility. To analyze intranuclear mobility, we used a variation of FRAP, termed strip-FRAP. Strip-FRAP was selected as the method of choice based on the even distribution of TR in the nucleus. In this method, a small strip through the nucleus is photobleached and mobility is monitored by the recovery of fluorescent proteins into the bleached strip.

To confirm the validity of our FRAP experiments, we first employed a series of strategies aimed at improving accuracy. The observed diffusional rate of TR from pilot studies emphasized the efficacy of strip-FRAP to take advantage of horizontal scan frequency. In this set-up, a “stimulation line” region of interest (ROI) was positioned within the nucleus of a cell and served as the targeted region for 1 s of photobleaching. Prior to data collection, several constraints were considered and applied to our strip-FRAP setup, which have previously been advised by others (Weiss, 2004; Yang et al., 2010). First, to ensure sufficient bleaching along the z-axis, we conducted strip-FRAP on fixed cell samples and acquired 3-dimensional “z-stacks” to quantify the bleaching depth (data not shown). Here, we consistently confirmed bleaching depths greater than the cell’s z-dimensional width and thus, verified a bleach depth greater than the cell width along the z-axis. Second, to minimize for any error created by lack of uniformity in diffusion from the x and y planes, sample selection was confined to nuclei with as symmetrical morphology as HeLa cell nuclei allowed. Third, brief studies were completed using ROIs at variable regions in nuclei to conclude that TR was evenly and diffusely distributed within nuclei. To further account for this, the stimulation/bleaching ROI was adjusted

to a symmetrical position in the nuclei along the midline. Similarly, cell/nucleus selection took into consideration the relative bleach area to total cell volume ratio. Finally, to reduce the concentration of protein diffusing into the bleach region during the bleaching phase, we combined resonant scanning and the lowest possible bleach interval allowable for considerable photobleaching. These adjustments allowed for a frame rate of ~15 images per second. PFS was also applied to stabilize the sample's focal position during FRAP and compensated for otherwise needing to increase pinhole aperture to account for drift. FRAP results with displaced ROIs from the intended positioning in nuclei, due to drift, that occurred pre-bleach, and therefore couldn't be accounted for with post-analysis tracking adjustments, were discarded.

After validating the use of strip-FRAP, we first compared cells expressing mCherry-TRα1 to cells co-expressing mCherry-TRα1 and GFP-MED1 simultaneously (Fig 4A). The resulting fluorescence intensity data were normalized using Eq. 1. Data normalization then allowed us to determine the recovery rate, mobile fraction, immobile fraction, and estimated T_{half} based on the FRAP curves. For TRα1 co-expressed with MED1, we observed no significant difference in any of these variables ($p > 0.05$) (Fig 4B, Table 1).

In contrast, when conducting the same MED1 cotransfection assay with TRβ1, there was a significant reduction in the estimated T_{half} and mobile fraction of TRβ1, and thus an increase in the immobile fraction (Table 1). p-values for mobile fraction/immobile fraction and estimated T_{half} were 0.007 and 0.035,

respectively. mCherry-TRβ1 alone had a faster influx of signal directly after photobleaching resulting in close to complete recovery, while co-overexpressed TRβ1 did not show complete recovery (Fig. 5).

Knockout of MED1 promotes export of TRα1 and TRβ1 into the cytoplasm. Given that MED1 is required for the expression of TR-regulated genes (Ito et al., 2000; Malik et al., 2004), we hypothesized that MED1 helps to anchor TR in the nucleus. Our findings from overexpression of MED1 suggest that MED1 indeed plays a role in nuclear retention of TR subtypes. To build on our findings, we chose to analyze TR's intracellular distribution in the absence of MED1. We anticipated that a greater cytoplasmic population of TR would be observed in null MED1/TRAP220$^{-/-}$ MEFs compared to wild-type MED1/TRAP220$^{+/+}$ MEFs. These cell lines were provided by the Roeder lab who previously verified successful knockout of MED1 (Ito et al., 2000). Our Western blots to confirm knockout of MED1 in the TRAP220$^{-/-}$ cells were unsuccessful, likely because of time constraints for optimizing extraction and transfer of this very large protein (data not shown).

After transfecting the null and wild-type MEF cell lines with TR expression plasmids, nucleocytoplasmic distribution was quantified using the previously described scoring method. Here, we show that mCherry-tagged TRα1 (Fig. 6) and TRβ1 (Fig. 7) have a significantly greater cytoplasmic population (lower N/C ratio) in TRAP220$^{-/-}$ MEFs, relative to their distribution patterns in TRAP220$^{+/+}$ cells ($p<0.01$), suggesting that in the absence of MED1, nuclear retention of TR

is decreased. Not surprisingly, given the primarily cytoplasmic distribution of v-ErbA in wild-type cells, no significant change in localization of v-ErbA was apparent in the TRAP220$^{-/-}$ MEFs ($p = 0.30$) (Fig. 8).

Phosphorylation of endogenous MED1 does not affect localization of TRα1 and v-ErbA, but T3-induced MED1 phosphorylation reduces TRβ1 nuclear retention. As discussed earlier, it has been shown that MED1 is phosphorylated at specific threonine residues, and that phosphorylated MED1 is a more potent coactivator of TR-regulated genes (Pandey et al., 2005; Belakavadi et al., 2008). Based on our analysis of TR's nucleocytoplasmic distribution in the presence and absence of MED1, we predicted that phosphorylated MED1 would increase retention of TR in the nucleus. To test this prediction, cells were incubated in charcoal-stripped FBS media depleted of T3 and growth factors. We then stimulated the MAPK-ERK signaling pathway to trigger phosphorylation of MED1 using EGF or T3 (Pandey et al. 2005), and compared the resulting distribution patterns of TR to that of T3-depleted (-T3) cells.

Contrary to our predictions, supplementing transfected cells with EGF had no effect on localization of mCherry-tagged TRα1, TRβ1, or v-ErbA ($p>0.05$ for each) (Figs. 9 – 11). Treating TRα1 and v-ErbA-transfected cells with T3 yielded similar outcomes. However, cells transfected with TRβ1 responded to T3 treatment by, unexpectedly, displaying a significant shift in TRβ1 towards the cytoplasm ($p<0.001$) (Fig. 10). The N/C values, before normalization, decreased from ≈ 6.5 to ≈ 4.0 with T3 treatment. It is known that proteasome-mediated

degradation of TR is more rapid in the presence of T3 (Bondzi et al., 2011), so it is possible that this change in distribution pattern results from altered protein turn-over, rather than direct interactions with MED1.

Phosphorylation of MED1 has minimal effect on TRα1 and TRβ1 mobility. Based on the finding that phosphorylation of MED1 enhances its interaction with TR (Belakavadi et al., 2008; Pandey et al., 2005), we predicted that inducing this modification in MED1 would decrease intranuclear mobility of TR, as assessed by Strip-FRAP. Here, we found that the mobility of both TRα1 and TRβ1 was largely unaffected by treatment with either T3 or EGF. Intriguingly, however, when compared to T3-depleted conditions, we found that TRβ1 had a significant increase in its rate of recovery when treated with T3 ($p < 0.05$), whereas the mobile fraction of TRα1 in the presence of EGF decreased significantly ($p < 0.05$) (Table 1). Interpretation of these findings will require further investigation, as they suggest more complex interactions with other nuclear factors and signaling pathways.

Intranuclear mobility of GR confirms comparability of TR FRAP. Prior to this thesis research, mobility values for TR had not been previously defined. On the other hand, mobility of GR by FRAP assays has been extensively characterized (Carrero et al., 2003; Groeneweg et al., 2014; Meijsing et al., 2007). Previous characterization of GR allowed us to assess the comparability of our assay. To compare our own results of GR mobility against previously reported values, we treated cells expressing GFP-GR with dexamethasone to induce a nuclear

population of GR. FRAP was then performed and analyzed. While others have reported halftimes of greater than a few seconds for GR, we found that on average, GR's halftime was ~0.74 seconds. Thus, our initial interpretation was that we were over-estimating mobility dynamics of proteins using our FRAP design. However, individual analysis of GR trials revealed two distinct populations of GR provoking a bimodally skewed distribution of our data. This phenomenon has recently been reported by a similar analysis of GR FRAP (Groeneweg et al., 2014). One GR state demonstrated a relatively fast halftime (~ 0.6 s) while another was much slower (~3.0 s) (Table 1). The latter is depicted in Figure 14A, and the former (data not shown) is both graphically and visually analogous to TRα1 or TRβ1 (see Figures 4 and 5).

To examine additional parameters impacting intranuclear mobility, we also used FRAP to analyze a previously constructed expression vector containing TR's hinge domain NLS (Mavinakere et al., 2012), which is abbreviated here as GFP-GST-GFP-NLS. Of the different attributes, the vector encodes a GFP-GST-GFP-tag allowing us to visualize its movement. Knowing that the GFP-GST-GFP-NLS fusion protein lacks the DBD domain present in TR, we predicted that an isolated NLS of TR would display greater intranuclear mobility than TR. In support of this, the GFP-GST-GFP-NLS fusion protein had a significantly faster rate of recovery than any TR subtype ($p<0.001$) (Fig. 14B, Table 1). Therefore, we conclude the comparability of our strip-FRAP experimental set-up with prior reports for other transcription factors. However,

future analysis of diffusional coefficients and model-fitting is needed to provide further support for this conclusion.

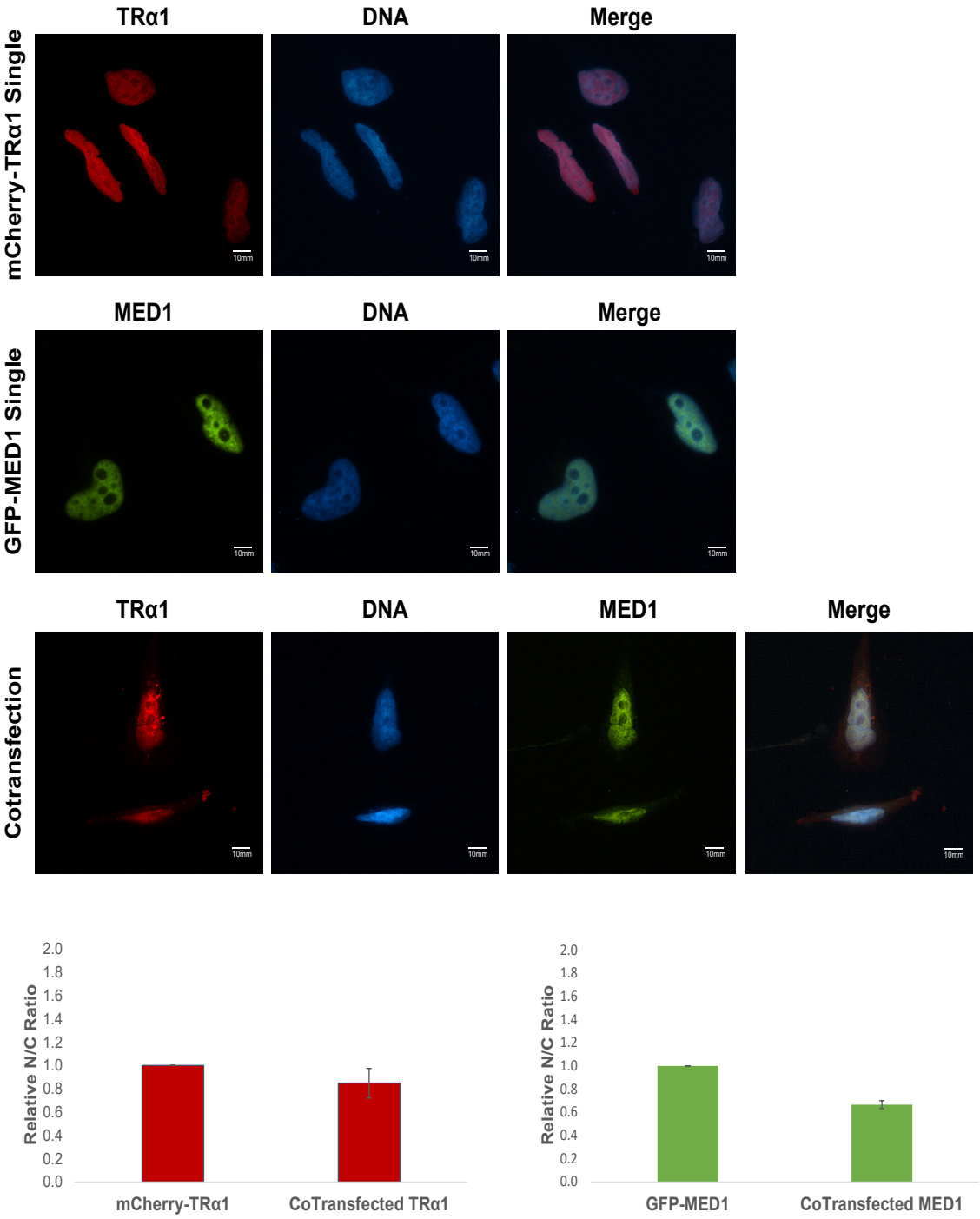

Figure 1. Overexpression of MED1 does not alter the nucleocytoplasmic distribution of TRα1. HeLa cells were transfected with either mCherry-TRα1 or cotransfected with mCherry-TRα1 and GFP-MED1. 24h post-transfection cells were analyzed using fluorescence microscopy. N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in single-transfected cells. (Bottom) Compared to transfecting TRα1 alone, co-overexpression with MED1 yielded no significant change in intracellular distribution of TRα1 ($p > 0.05$). However, nuclear retention of MED1 was significantly reduced from cotransfection with TR α1 ($p < 0.001$).

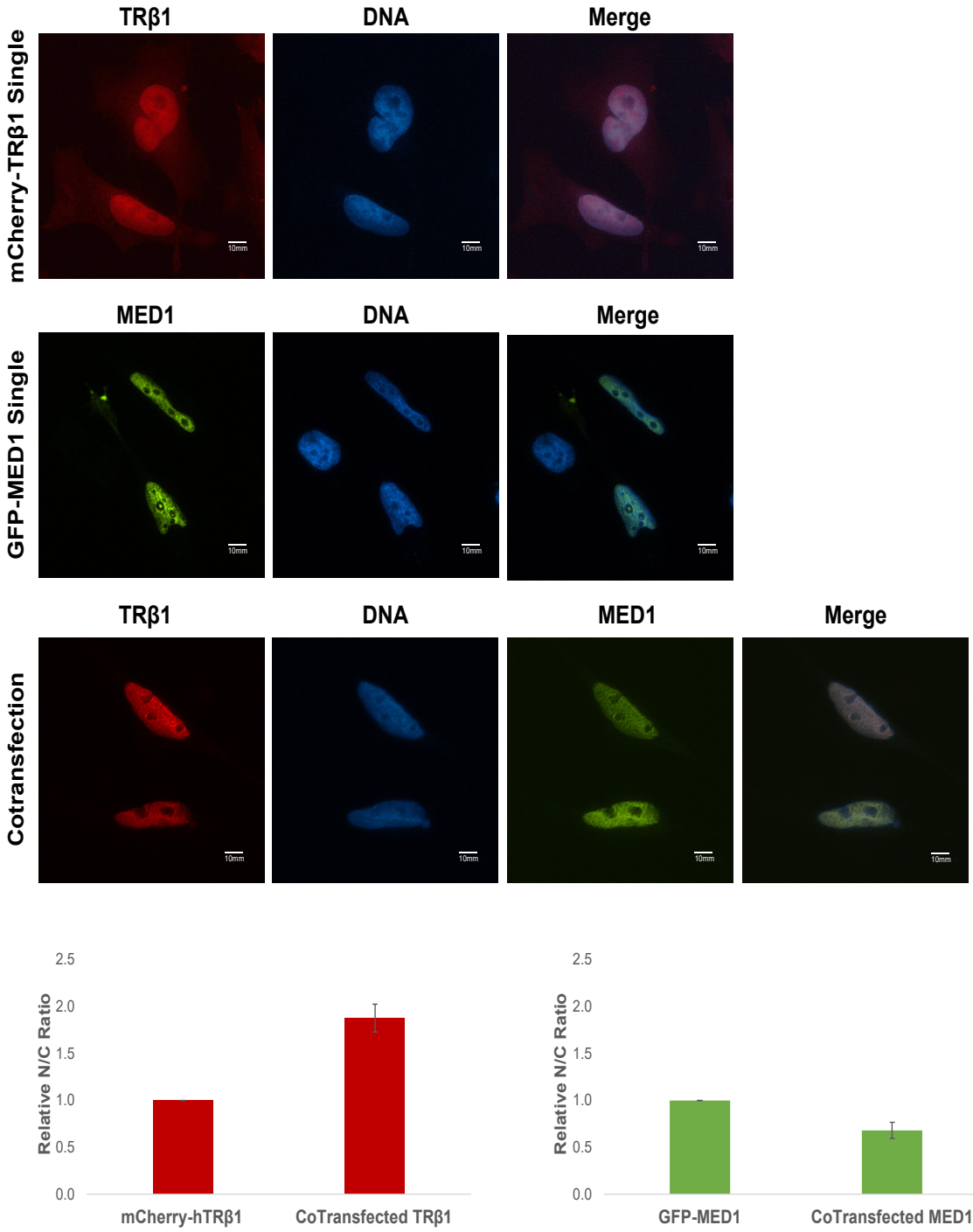

Figure 2. Overexpression of MED1 increases the nuclear retention of TRβ1. HeLa cells were transfected with either mCherry-TRβ1 or cotransfected with mCherry-TRβ1 and GFP-MED1. 24h post-transfection cells were analyzed using fluorescence microscopy. N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in single-transfected cells. (Bottom) Compared to transfecting TRβ1 alone, co-overexpression with MED1 increased the nuclear localization of TRβ1 ($p < 0.01$). Additionally, the retention of MED1 significantly decreased ($p < 0.05$).

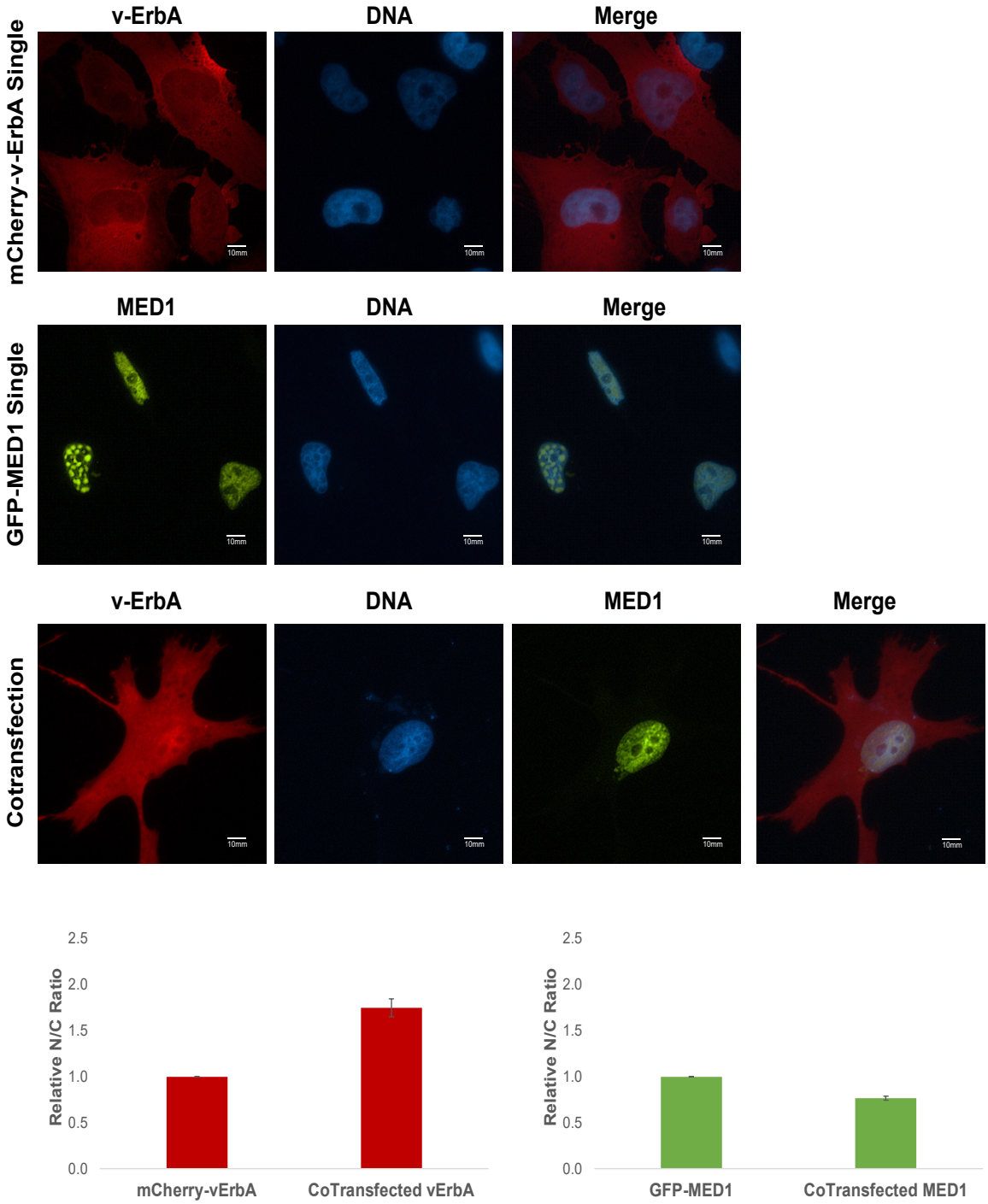

Figure 3. Overexpression of MED1 increases the nuclear retention of v-ErbA. HeLa cells were transfected with either mCherry-v-ErbA or cotransfected with mCherry-v-ErbA and GFP-MED1. 24h post-transfection cells were analyzed using fluorescence microscopy. N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in single-transfected cells). (Bottom) Compared to transfecting v-ErbA alone, co-overexpression with MED1 increased the nuclear localization of v-ErbA ($p < 0.01$). Additionally, the retention of MED1 significantly decreased ($p < 0.001$).

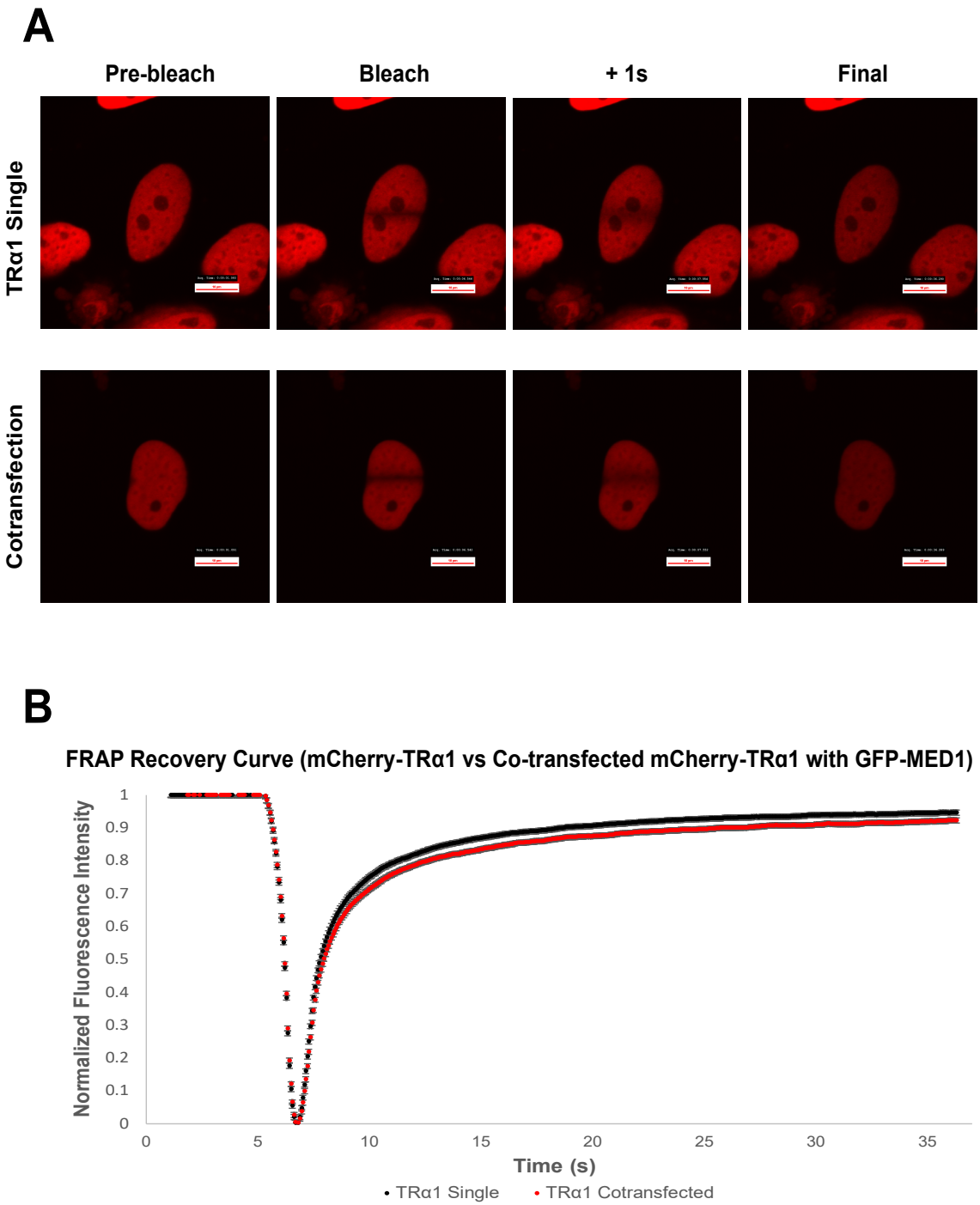

Figure 4. Co-transfecting TRα1 with GFP-MED1 does not alter the intranuclear mobility of TRα1. HeLa cells were transfected with either mCherry-TRα1 or cotransfected with mCherry-TRα1 and GFP-MED1. Strip-FRAP was conducted on nuclei from 20 separate cells using a stimulation bleaching line near the midline of the nuclei. 3 biological replicates were performed (n=3). Data were normalized and compared between TRα1 transfected alone (TRα1 single) and Cotransfected TRα1 (Cotransfection). **(A)** Nuclei prior to bleach (pre-bleach), directly after bleaching terminated (bleach), 1 second post-bleach (+1s), and at the end of the recovery (final). Scale bar is defined as 10μm. **(B)** Graphical representation of the FRAP recovery curves acquired from the 3 biological replicates. Comparison between TRα1 expressed alone and TRα1 cotransfected is shown

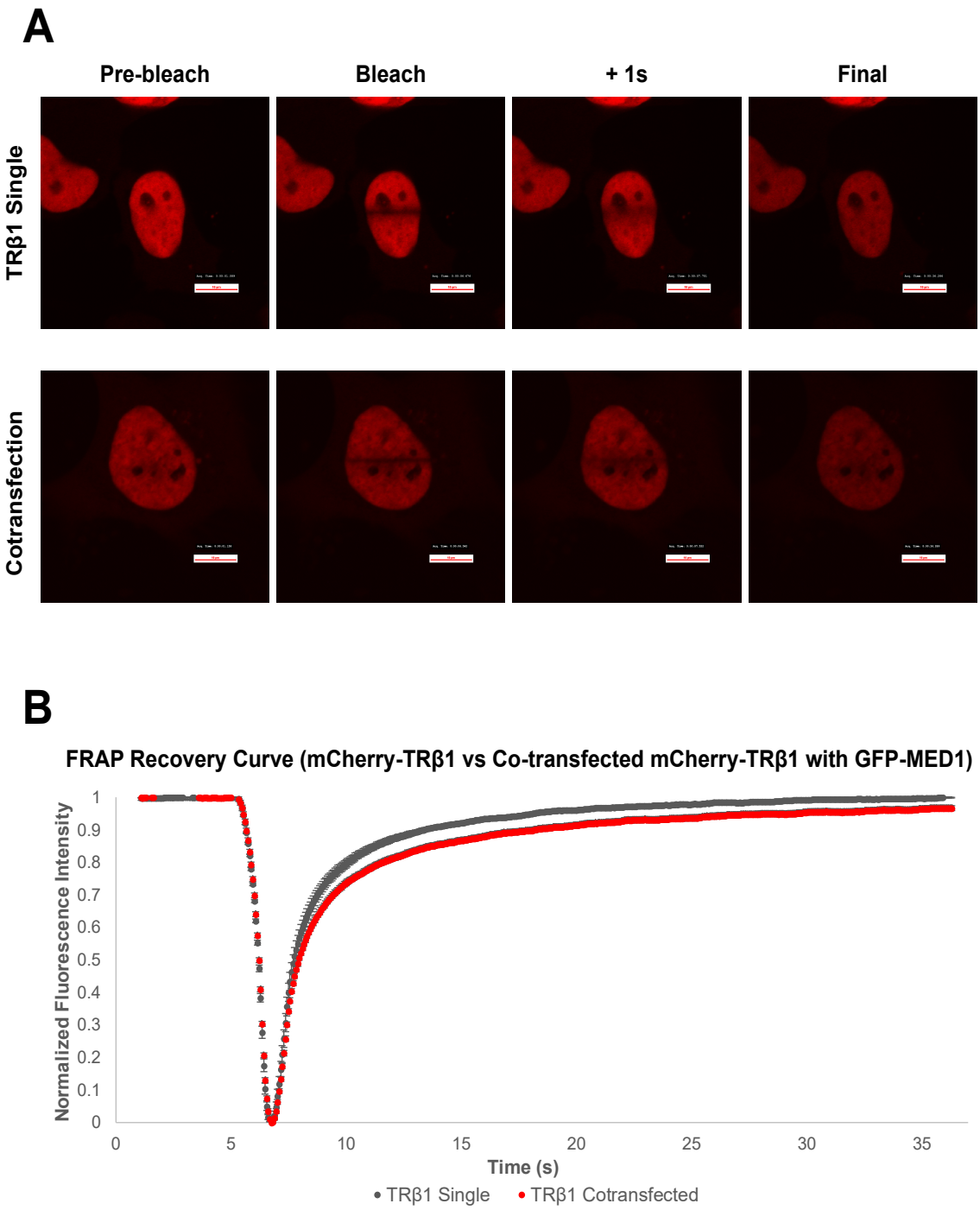

Figure 5. Co-transfecting TRβ1 with GFP-MED1 reduces the halftime and mobile fraction of nuclear TRβ1. HeLa cells were transfected with either mCherry-TRβ1 or cotransfected with mCherry-TRβ1 and GFP-MED1. Strip-FRAP was conducted on nuclei from 20 separate cells using a stimulation bleaching line near the midline of the nuclei. 3 biological replicates were performed (n=3). Data were normalized and compared between TRβ1 transfected alone (TRβ1 single) and Cotransfected TRβ1 (Cotransfection). **(A)** Nuclei prior to bleach (pre-bleach), directly after bleaching terminated (bleach), 1 second post-bleach (+1s), and at the end of the recovery (final). Scale bar is defined as 10μm. **(B)** Graphical representation of the FRAP recovery curves acquired from the 3 biological replicates. Comparison between TRβ1 expressed alone and TRβ1 cotransfected is shown

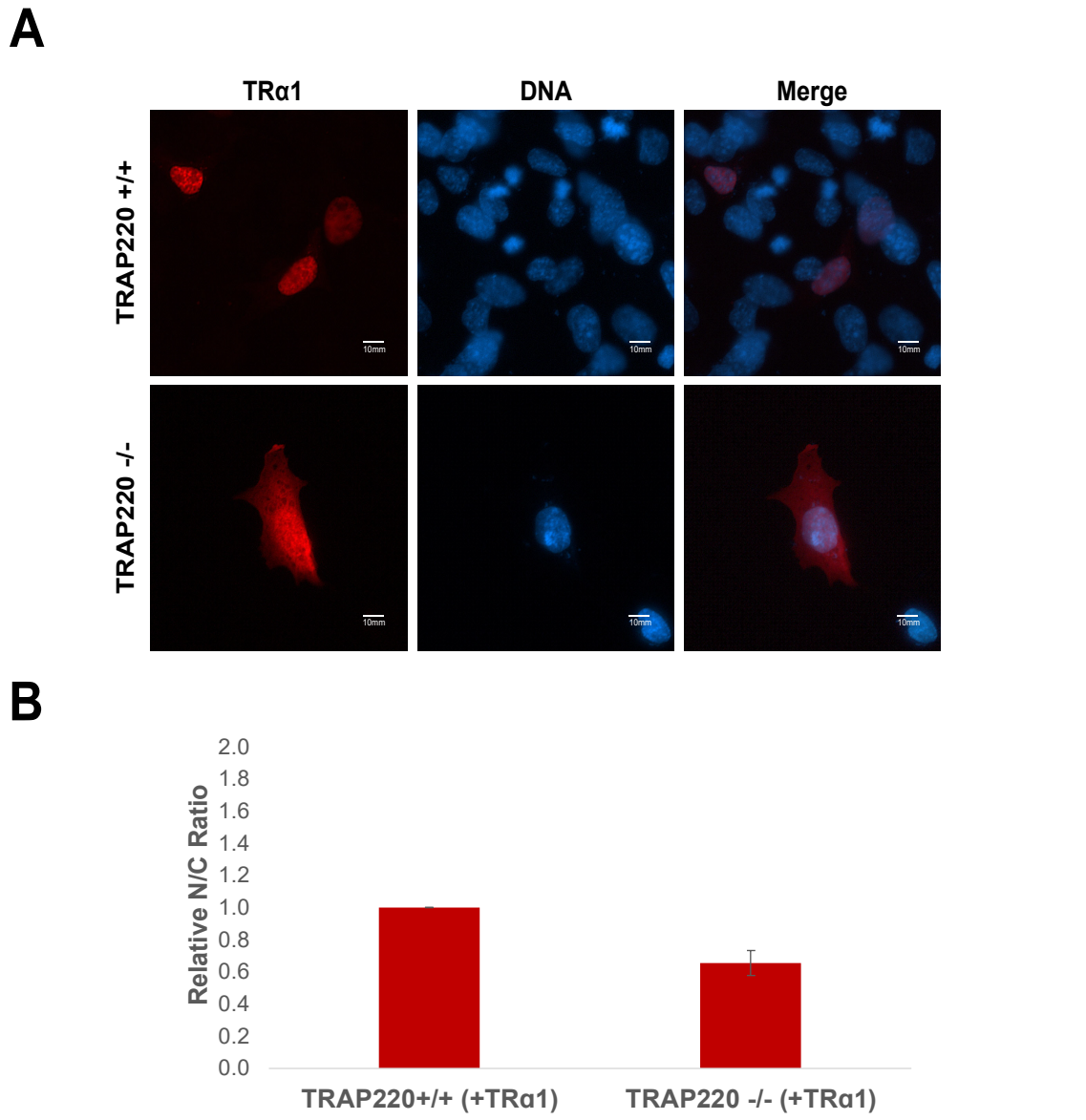

Figure 6. Knockout of MED1 decreases the nuclear retention of TRα1. (A) Mouse embryonic fibroblasts (MEFs) were transfected with mCherry-TRα1. 24h post-transfection cells were analyzed using fluorescence microscopy. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in wild-type cells). TRAP220 +/+ MEFs expressing TRα1 had a greater nuclear distribution of TRα1 while an increase in cytoplasmic distribution is observed in the TRAP220 -/- (top). Comparison of the N/C ratios showed a decreased nuclear retention in the TRAP220 -/- MEFs compared to the TRAP220 +/+ ($p < 0.05$).

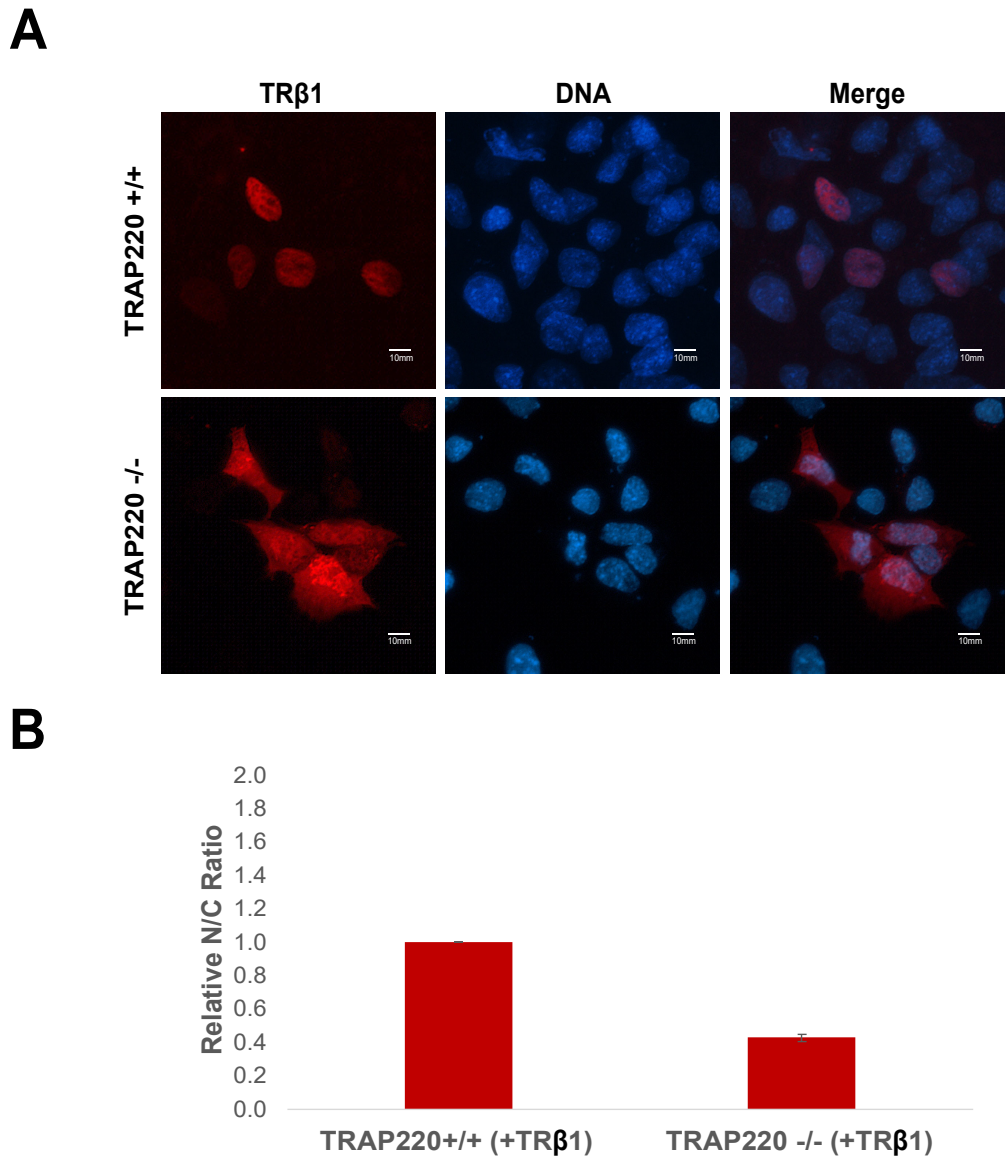

Figure 7. Knockout of MED1 decreases the nuclear retention of TRβ1. (A) Mouse embryonic fibroblasts (MEFs) were transfected with mCherry-TRβ1. 24h post-transfection cells were analyzed using fluorescence microscopy. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in wild-type cells. TRAP220 +/+ MEFs expressing TRβ1 had a greater nuclear distribution of TRβ1 while an increase in cytoplasmic distribution is observed in the TRAP220 -/- (top). Comparison of the N/C ratios showed a decreased nuclear retention in the TRAP220 -/- MEFs compared to the TRAP220 +/+ ($p = 0.001$).

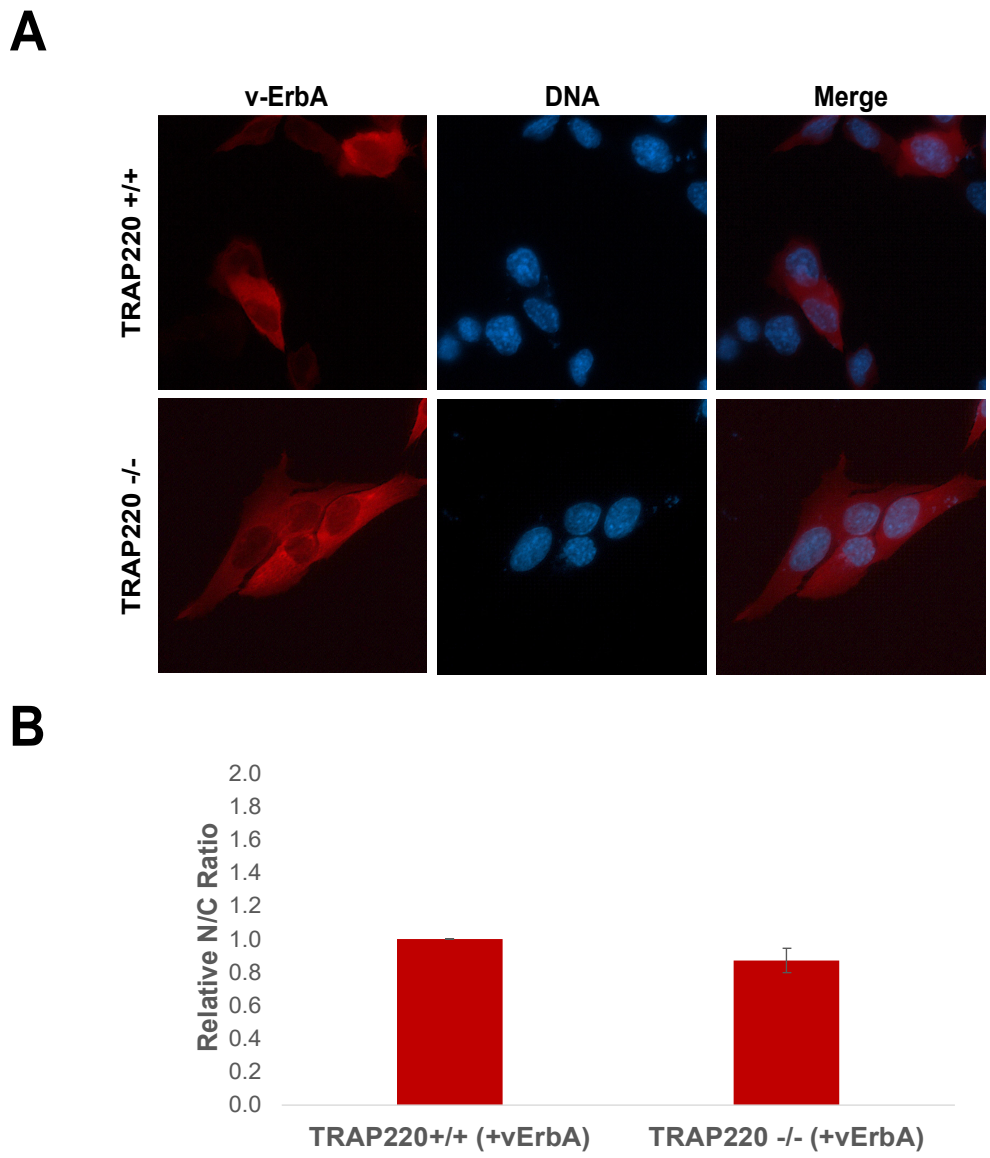

Figure 8. Knockout of MED1 has no effect on the nuclear retention of v-ErbA. (A) Mouse embryonic fibroblasts (MEFs) were transfected with mCherry-v-ErbA. 24h post-transfection cells were analyzed using fluorescence microscopy. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in wild-type cells). Both the TRAP220 +/+ and TRAP220 -/- MEFs expressing mCherry-v-ErbA exhibited a primarily cytoplasmic localization. Comparison of the N/C ratios for both MEFs revealed no significant difference in nuclear retention of v-ErbA ($p > 0.05$).

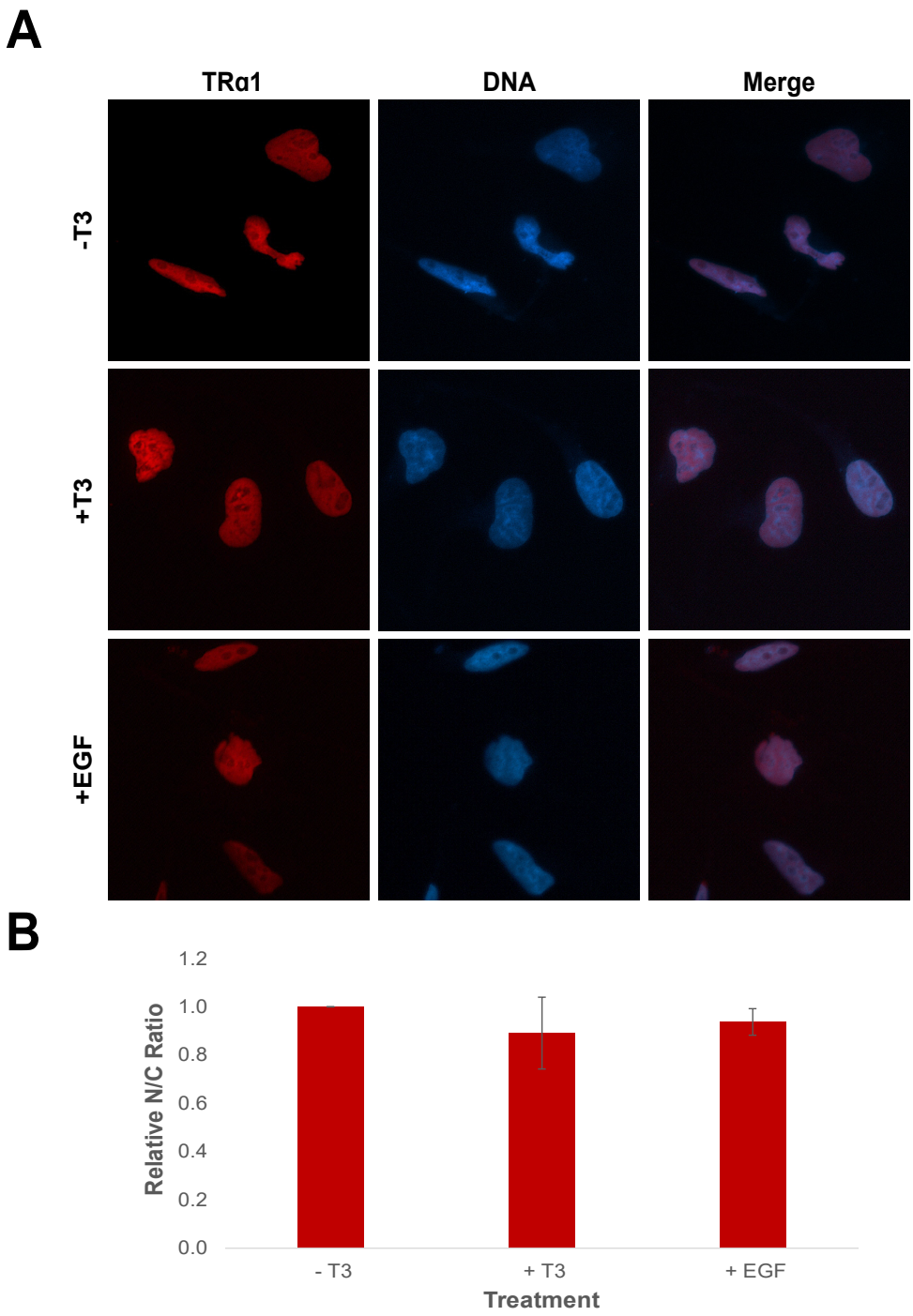

Figure 9. Phosphorylation of MED1 does not affect the nuclear retention of TRα1. (A) HeLa cells were transfected with mCherry-TRα1. 8h after transfection, cells were incubated in charcoal-stripped FBS and EGF or T3. 24 hours post-transfection, nucleocytoplasmic distribution was assessed. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in -T3 cells). Induction of MED1 phosphorylation with T3 and EGF did not have a significant effect on the retention of TRα1 ($p > 0.05$).

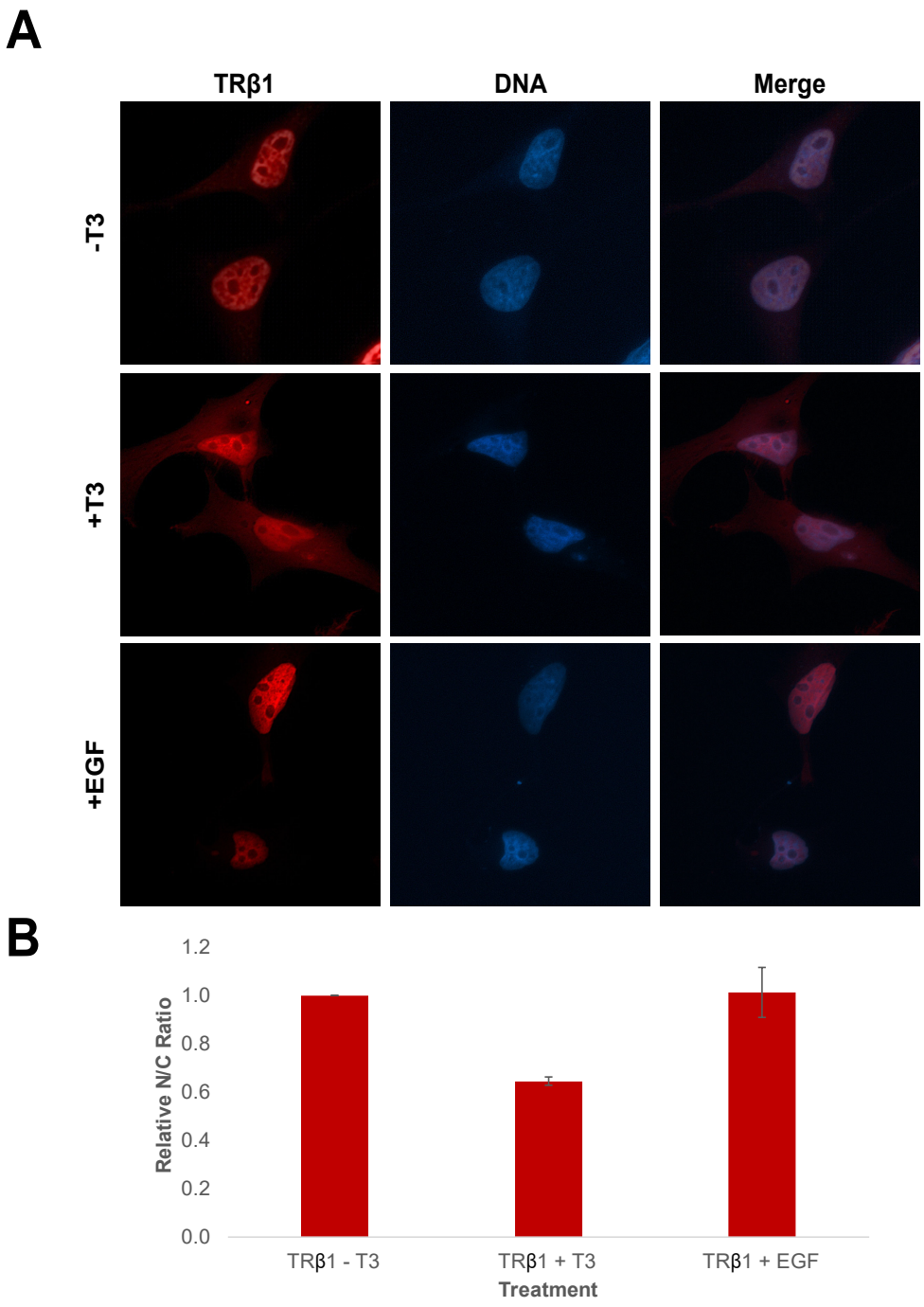

Figure 10. Phosphorylation of MED1 by T3 treatment increases amount of TRβ1 in the cytoplasm. (A) HeLa cells were transfected with mCherry-TRβ1. 8h after transfection, cells were incubated in charcoal-stripped FBS and EGF or T3. 24 hours post-transfection, nucleocytoplasmic distribution was assessed. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in -T3 cells). Induction of MED1 phosphorylation with both by EGF stimulation of MAPK-ERK signaling failed to have a significant effect on the retention of TRα1 ($p > 0.05$). However, treatment of TRβ1-expressing cells with T3 caused a decrease in nuclear retention, causing a shift of protein towards the cytoplasm ($p < 0.001$). Primarily nuclear localization of TRβ1 was

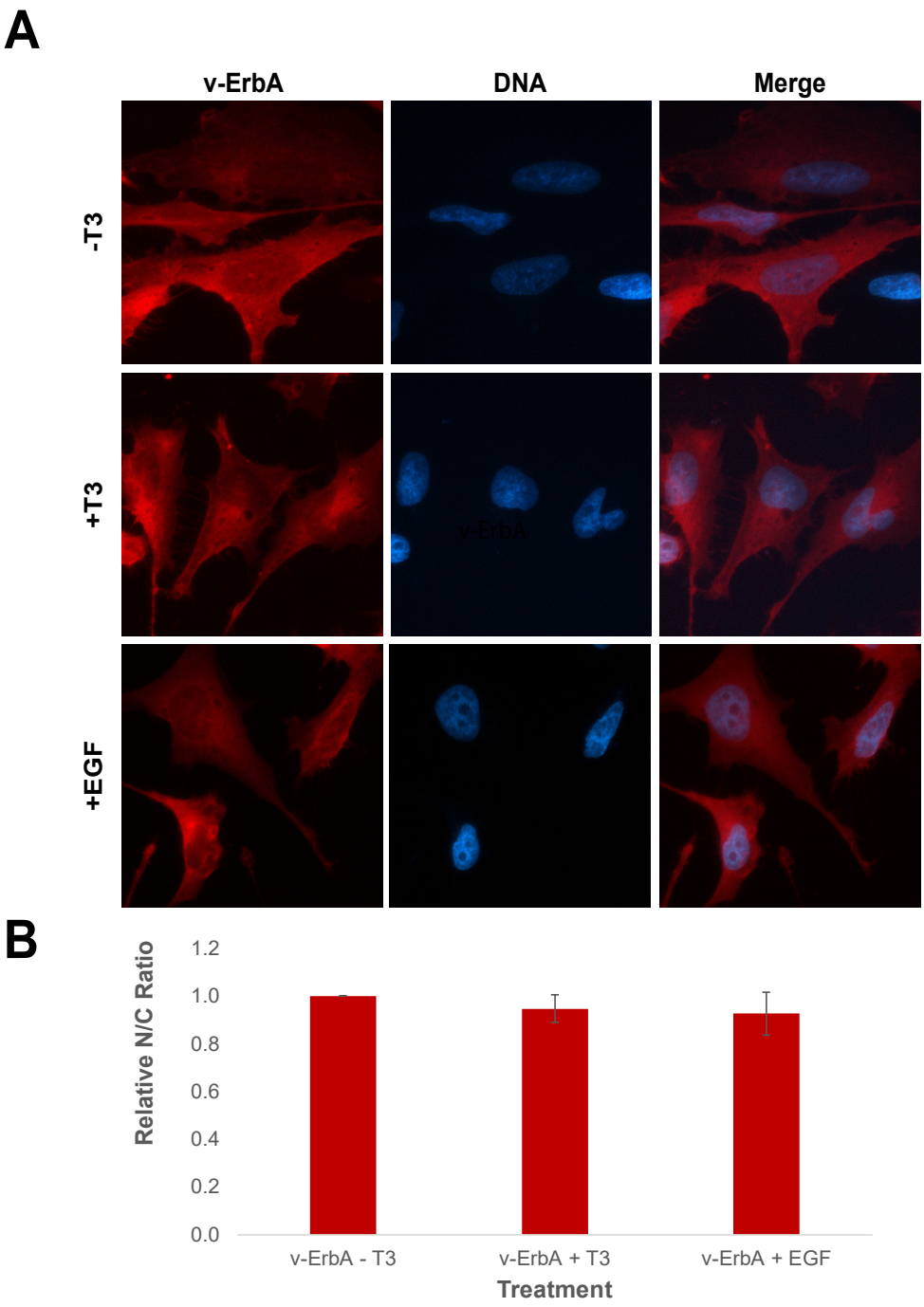

Figure 11. Phosphorylation of MED1 does not affect the nuclear retention of v-ErbA. (A) HeLa cells were transfected with mCherry-v-ErbA. 8h after transfection, cells were incubated in charcoal-stripped FBS and EGF or T3. 24 hours post-transfection, nucleocytoplasmic distribution was assessed. **(B)** N/C ratios were determined for 3 replicates of 100 cells and the average, relative N/C ratio was calculated (normalized to the distribution in -T3 cells). Induction of MED1 phosphorylation with both T3 and EGF did not have a significant effect on the retention of v-ErbA ($p > 0.05$).

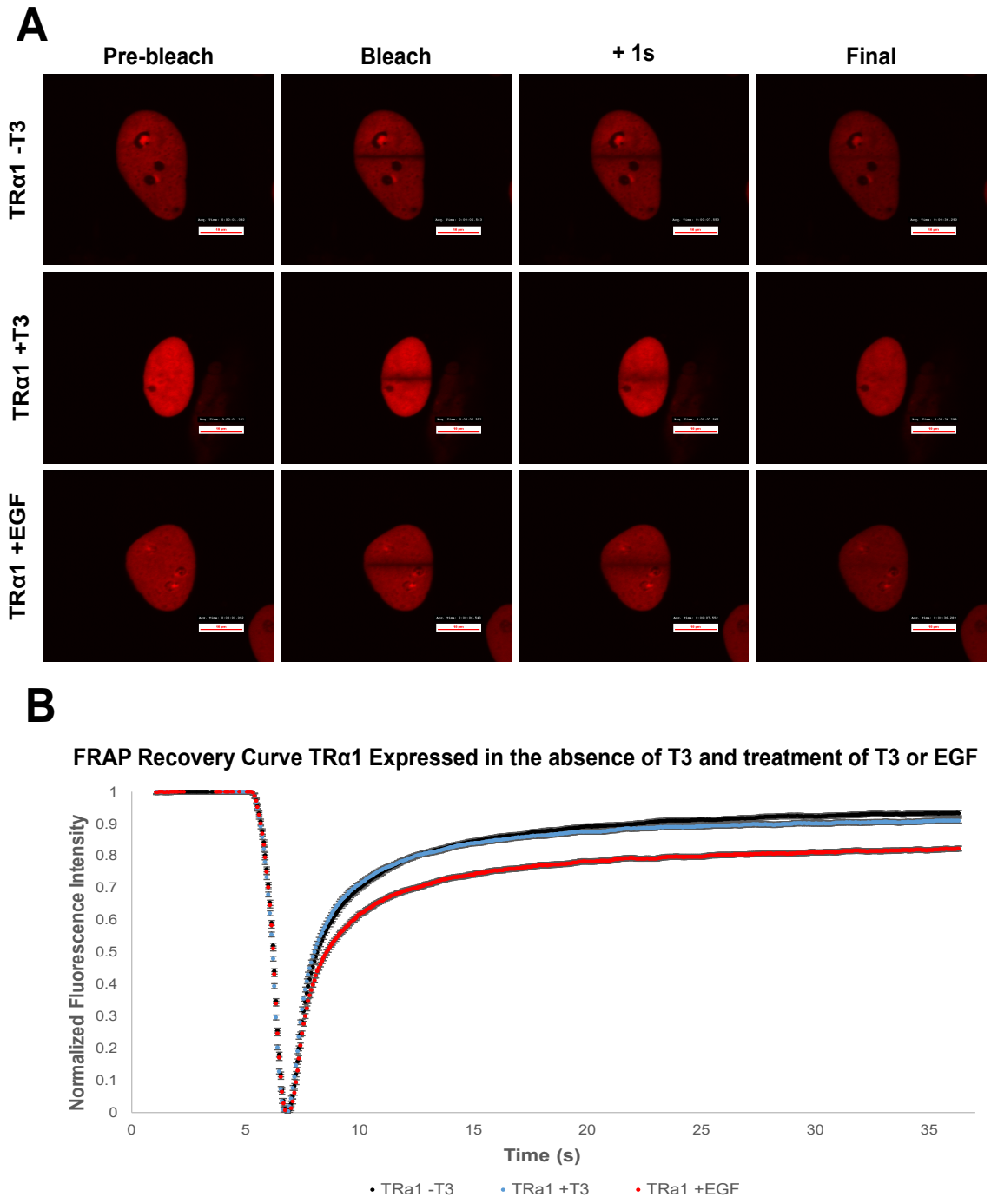

Figure 12. EGF-induced phosphorylation of MED1 reduces the mobile fraction of TRα1 HeLa cells were transfected with mCherry-TRα1. 8h after transfection, cells were incubated in charcoal-stripped FBS and EGF or T3. Strip-FRAP was performed on 3 biological replicates of 20 cells each. **(A)** Nuclei prior to bleach (pre-bleach), directly after bleaching terminated (bleach), 1 second post-bleach (+1s), and at the end of the recovery (final). Scale bar is defined as 10μm. **(B)** Graphical representation of the FRAP recovery curves acquired averages between the replicates Comparison between TRα1 -T3 (black), TRα1 +T3 (blue), and TRα1 +EGF (red) is displayed. Treatment with EGF caused a reduction in mobile fraction from ~93% in TRα1 -T3 to ~82% ($p = 0.01$).

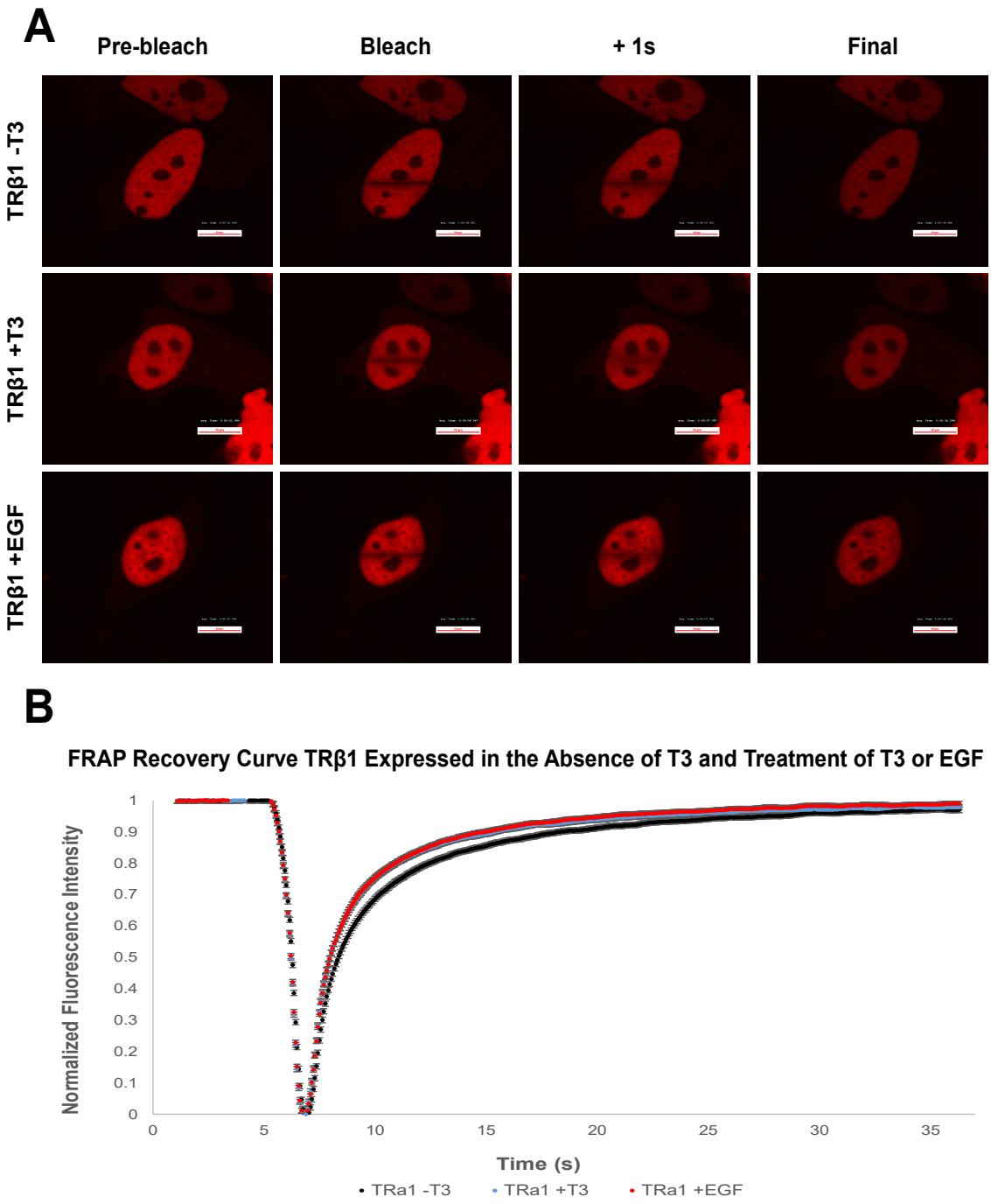

Figure 13. T3-induced phosphorylation of MED1 increases the rate of TRβ1 recovery. HeLa cells were transfected with mCherry-TRβ1. 8h after transfection, cells were incubated in charcoal-stripped FBS and EGF or T3. Strip-FRAP was performed on 3 biological replicates of 20 cells each. **(A)** shows nuclei prior to bleach (pre-bleach), directly after bleaching terminated (bleach), 1 second post-bleach (+1s), and at the end of the recovery (final). Scale bar is defined as 10μm. **(B)** Graphical representation of the FRAP recovery curves acquired averages between the replicates Comparison between TRβ1 -T3 (black), TRβ1 +T3 (blue), and TRβ1 +EGF(red) is displayed. Treatment with T3 caused an increase in the rate of TRβ1 recovery within the bleached region ($p < 0.05$).

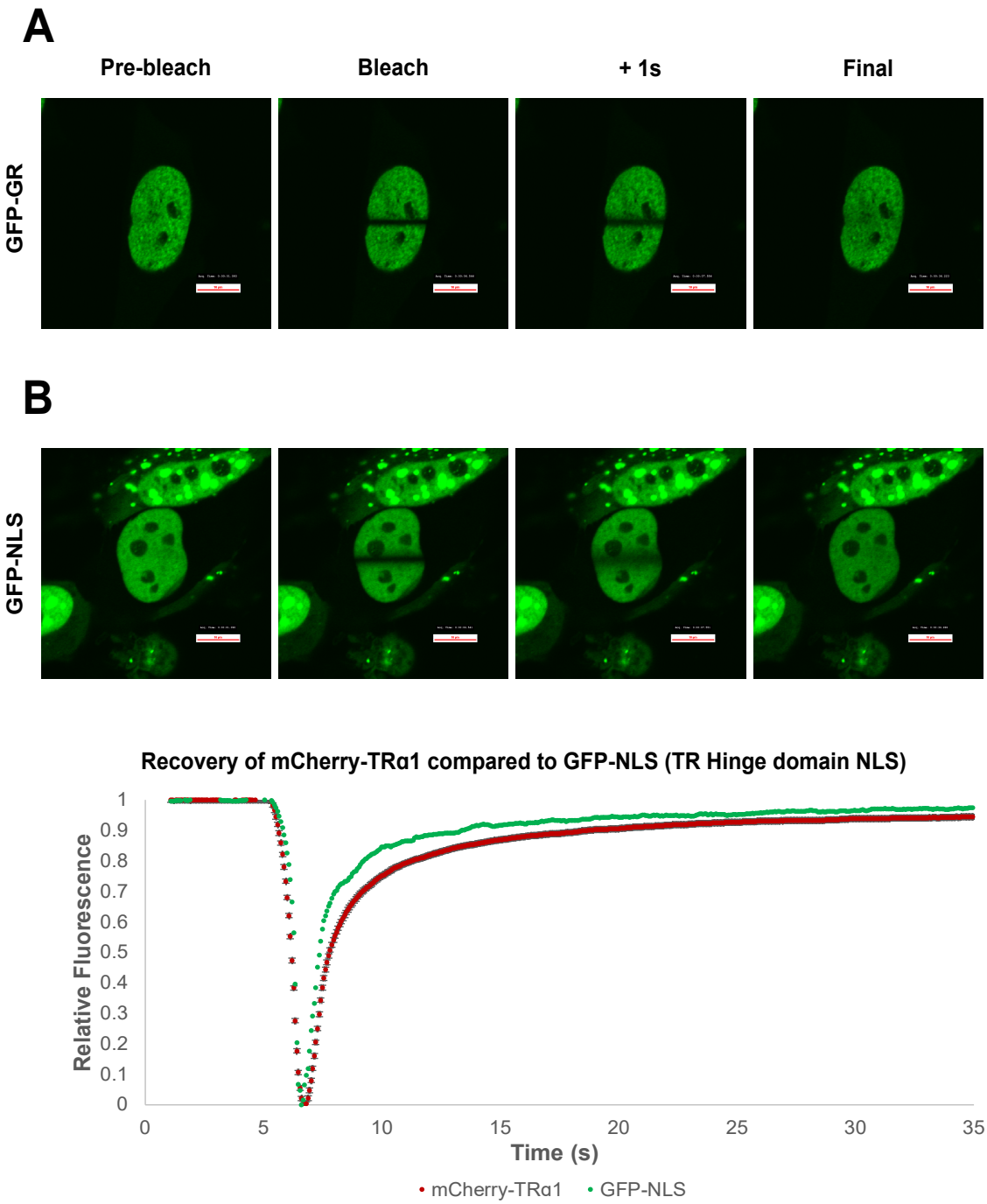

Figure 14. GFP-GR and GFP-NLS FRAP shows TRα1's NLS has greater mobility than full-length TRα1. HeLa cells were transfected with either mCherry-TRα1, GFP-GR, or GFP-NLS. Strip-FRAP was conducted on nuclei from 20 separate cells using a stimulation bleaching line near the midline of the nuclei. 3 biological replicates were performed (n=3). Data were normalized and compared. **(A)** shows nuclei expressing GFP-GR prior to bleach (pre-bleach), directly after bleaching terminated (bleach), 1 second post-bleach (+1s), and at the end of the recovery (final). Of two mobility states for GFP-GR, the slower is displayed here. **(B)** Graphical comparison between FRAP of mCherry TRα1 (red) and GFP-NLS (green). GFP-NLS has a significantly faster rate of recovery than native TRα1 ($p < 0.001$).

Treatment	Slope	Mobile Fraction	Immobile Fraction	Half-time
TRα1 Single	0.011 ± 0.0003	0.946 ± 0.03	0.054 ± 0.03	1.01s ± 0.18s
TRα1 Cotransfected	0.012 ± 0.0009	0.922 ± 0.05	0.078 ± 0.05	1.21s ± 0.42s
TRα1 -T3	0.013 ± 0.0014	0.932 ± 0.02	0.068 ± 0.02	1.26s ± 0.19s
TRα1 +T3	0.011 ± 0.0008	0.910 ± 0.02	0.089 ± 0.02	1.17s ± 0.21s
TRα1 +EGF	0.011 ± 0.0007	0.822 ± 0.07	0.178 ± 0.07	1.79 ± 0.92s
TRβ1 Single	0.012 ± 0.0007	0.999 ± 0.03	0.0006 ± 0.03	0.92s ± 0.08s
TRβ1 Cotransfected	0.013 ± 0.0006	0.968 ± 0.01	0.032 ± 0.01	1.20s ± 0.13s
TRβ1 -T3	0.014 ± 0.0007	0.970 ± 0.01	0.030 ± 0.01	1.37s ± 0.17s
TRβ1 +T3	0.013 ± 0.0004	0.976 ± 0.02	0.023 ± 0.02	1.10s ± 0.17s
TRβ1 +EGF	0.013 ± 0.0009	0.988 ± 0.01	0.012 ± 0.01	1.08s ± 0.12s

Table 1. FRAP measurements for TRα1 and TRβ1. Slope, mobile and immobile fraction, and halftime measurements for TRα1 and TRβ1 under various conditions. Data were calculated from FRAP curves for each separate condition. TR "Single" indicates cells expressing only exogenous TR, while "Cotransfected" indicates cells cotransfected with MED1, as well. MED1 phosphorylation assays are represented by the data for TRα1 or TR β1 ±T3/EGF.

DISCUSSION

In our prior studies, we showed that TR is capable of nucleocytoplasmic shuttling (Bunn et al., 2001). Our efforts further revealed the signal sequences that are responsible for TR's shuttling capability, as well as determining that TR follows both CRM1-dependent and CRM1-independent export pathways (Grespin et al., 2008; Mavinakere et al., 2012). Recently, we've identified the various exportins that facilitate CRM1-dependent export and the importins responsible for TR's entry into the nucleus (Roggero et al., 2016; Subramanian et al., 2015). Here, we extend our understanding of the fine balance between nuclear import, nuclear retention, and nuclear export of TR, by providing evidence that MED1 plays a key role in regulating the nuclear retention of TR variants.

We showed that overexpression of MED1 in HeLa cells leads to a greater nuclear distribution of TRβ1 and v-ErbA, and decreases the T_{half} and mobile fraction of TRβ1. On the other hand, knockout of MED1 in MEFs resulted in a greater cytoplasmic distribution of TRα1 and TRβ1 relative to the distribution pattern in cells expressing MED1. We also show that T3-induced phosphorylation of MED1 causes a reduction in nuclear retention of TRβ1 and, also, increases the intranuclear mobility of TRβ1 relative to T3-depleted conditions. Collectively, our results provide evidence that MED1 plays a definitive role in regulating nuclear retention of TR subtypes.

MED1-regulated TR retention is dependent on MED1

Prior to our analysis, MED1 had previously been shown to play an essential role in TR-controlled gene expression. In fact, knockout of MED1 causes dysfunction in TR's regulation to an extent that resulted in embryonic lethality in mice (Ito et al., 2000). The significance of the interaction between MED1 and TR led us to propose that MED1 is also an important component governing TR's nuclear retention. Using a combination of strip-FRAP and nucleocytoplasmic scoring, we determined that MED1 is involved in TR nuclear retention. The direct interaction between TR subtypes and MED1 is supported by our findings from overexpression assays. When overexpressing MED1 by itself, distinct aggregates of MED1 were spread diffusely throughout the nucleus. However, when either TRα1, TRβ1, or v-ErbA was co-expressed, the MED1 aggregates no longer formed, suggesting that the MED1 aggregates were resolved following interaction with the exogenous TR.

Interestingly, MED1 overexpression and knockout led to different results for TRα1, TRβ1, and v-ErbA. Overexpression of MED1 caused TRβ1 and v-ErbA to shift towards significantly greater nuclear retention. TRβ1 and v-ErbA both have a higher detectable cytosolic population of protein relative to TRα1. This difference in distribution is thought to result, in part, from TRα1's NLS-2 in the A/B domain which is not present in TRβ1 and is mutated in v-ErbA (Mavinakere et al., 2012). As noted in the results, we conclude that our nucleocytoplasmic scoring method was more efficient in capturing a difference in retention for TRβ1 and v-ErbA rather than TRα1, because of TR's primarily nuclear localization. Likewise, when performing similar analysis with MED1

knocked out, where we anticipated a reduction in retention and a greater distribution in the cytoplasm, no significant change in v-ErbA, a primarily cytosolic protein, was observed. However, for TRα1 and TRβ1, which are primarily nuclear in wild-type cells, we did observe a cytosolic shift in TRα1 and TRβ1 distribution in MED1-null cells.

As mentioned, TRα1 and TRβ1 carry NES-H12 in their LBDs, a motif that is naturally deleted from v-ErbA (Mavinakere et al., 2012). Extrapolating off our current MED1 knockout results, we propose a model involving competition between MED1 and exportins 4, 5, or 7 to balance nuclear retention and export of TR. Therefore, by removing MED1 from this competition in TRAP220$^{-/-}$ MEFs, it's likely that the greater cytoplasmic distribution of TR relative to TRAP220$^{+/+}$ MEFs that we observed is due to increased interaction between NES-H12 of TRα1 and TRβ1 and exportins 4, 5, or 7. On a different note, we've also shown that v-ErbA not only localizes predominantly to the cytoplasm, but that it also dimerizes with TRα1 and causes a fraction of TRα1's nuclear population to mislocalize to the cytoplasm (Bonamy et al.; Bunn et al., 2001). While TRα1 has been shown to interact directly with MED1, v-ErbA's interaction with MED1 is still yet to be tested. Considering this, we hypothesize the possibility of an indirect interaction of v-ErbA with MED1 which causes the observed shift in v-ErbA towards the nucleus when MED1 is overexpressed. Ultimately, since v-ErbA is still dimerizing with the low endogenous TRα1 concentration in the nucleus, it's possible that overexpressing MED1 leads to greater association

with this dimer through binding TRα1 and effectively reduces the nuclear export of v-ErbA.

Phosphorylated MED1 variably affects retention of TRα1 and TRβ1

Our interest in phosphorylation of MED1 was considered in light of evidence for MED1-stimulated increases in TR-dependent transcription (Pandey et al., 2005). Interestingly, MED1 phosphorylation also leads to the upregulation of MED1 directly, which ultimately accelerates various forms of cancer (Jin et al., 2013; Vijayvargia et al., 2007). Contrary to our prediction that phospho-MED1 would enhance nuclear retention of TR, we found that after stimulating phosphorylation of MED1, the retention of TRα1, TRβ1, and v-ErbA was unaffected. As an exception to this trend, addition of T3 caused decreased nuclear retention of TRβ1 compared to the T3-depleted conditions in our nucleocytoplasmic scoring assay. On the contrary, we previously reported that addition of T3 to *Xenopus* (amphibian) oocytes resulted in greater nuclear retention of TRα1 but this effect was not observed in mammalian cells cultured in the presence or absence of T3 (Bunn et al., 2001). The disagreement between our past findings and the results in this study suggests different responses to thyroid hormone in *Xenopus* models compared to our HeLa-mammalian cell approach. Regardless, the variability in response between TRα1 and TRβ1 is puzzling and requires further analysis. We recently conducted a pilot study using longer incubation periods for T3 and EGF which provoked a sharper decrease in mobile fraction (data not shown). However,

cells were incubated significantly longer than others have used to induce MED1 phosphorylation (Belakavadi et al., 2008; Kim et al., 2017; Pandey et al., 2005). Questions surrounding cytotoxicity and cell viability using this extended treatment must be addressed in future work to justify the cogency of an alternative approach.

In addition to the scoring analysis, using FRAP to probe the effects of phospho-MED1 TR mobility yielded similar unexpected outcomes. In disagreement with our predictions, T3-stimulated phosphorylation of MED1 caused an increase in the mobility of TRβ1. This result was consistent with a reduction in TRβ1 nuclear retention from nucleocytoplasmic scoring under the same conditions. Similarly, MED1 phosphorylation had minimal effect on the nuclear mobility of TRα1. However, we did observe that EGF treatment independently affected TRα1's dynamics, although, studies have shown *both* EGF and T_3 induce MED1 phosphorylation with similar efficiency (Belakavadi et al., 2008). Aside from altering the mechanics of our experiment, it is possible that intrinsic cell-control mechanisms are protecting against the harmful effects of MED1 upregulation. Phosphorylation of MED1 has been shown to increase its half-life and thus contribute to its overexpression (Belakavadi et al., 2008). Therefore, its plausible that in the presence of a greater phospho-MED1 population, control mechanisms are induced that subsequently degrade the protein, as well.

Recently, however, this stabilization of MED1 has generated much interest for its contribution to the development and progression of several cancers, including breast, lung, and prostate cancer (Chen et al., 2011; Cui et al., 2012; Jin et al., 2013; Kim et al., 2012; Vijayvargia et al., 2007). MED1 concentration and phosphorylation are surveilled by microRNA-205 (miR-205), a potent inhibitor of MED1 and its phosphorylation-induced stabilization, and which assists in MED1 mRNA degradation (Hulf et al., 2012; Jin et al., 2013; Mouillet et al. 2012). Therefore, it has been proposed that miR-205 regulates the proper intracellular concentration of MED1. Interestingly, miR-205 is constitutively upregulated in cervical cancer cells (Xie et al., 2012). Based on the origin of HeLa cells, this would point to the prospect of these cells upregulating the production of miR-205, under standard conditions. By artificially stabilizing the half-life of MED1 through phosphorylation, we may also have been simultaneously inducing an even higher concentration of the endogenously upregulated miR-205. Effectively, this would amplify the regulation applied to HeLa-endogenous MED1. Targeted for phosphorylation through both ERK and AKT signaling, phospho-MED1 also bridges these pathways to several downstream targets which are typically kept under tight regulation and may contribute to a rapid degradation of the phosphoprotein (Jin et al., 2013). Taken together, this would help to explain a difference between the observations made with our overexpression and phosphorylation assays due to our reliance on endogenous MED1 concentration for analysis of the

phosphorylation assay rather than introducing an influx to counteract miR-205, as we may have unknowingly done with the overexpression studies.

In summary, either overexpressing MED1 during phosphorylation analysis or performing the phosphorylation assays in an alternative cell line, as others have done (Chen et al., 2011; Jin et al., 2013; Kim et al., 2017), will remove the possibility of this complication. Additionally, comparing the relative concentration of miR-205 in untreated, MED1-overexpressed, and upregulated phospho-MED1 cells will provide additional insight into how cells balance MED1 levels to regulate downstream targets such as TR and its nucleocytoplasmic distribution.

Our findings with MED1 provide an exciting prospect toward understanding TR disease pathogenesis. Reports show that some of the several mutations in TRβ1 that contribute to RTH occur in the LBD, and more specifically, within helix 12 (Yen et al., 2001). In combination with knowing that MED1 interacts with helix 12 of TR and our findings reported here, it is possible that such a mutation may disrupt the MED1-TR interaction and generate mislocalization of the receptor. However, one study revealed that MED1 is curiously only found in a small subset of cells (Zhang et al., 2005). This suggests a distinct role for MED1 in TR retention for some cell types, but additional mechanisms may be in place to adequately maintain its localization. Regardless, we conclude here that TR's nuclear retention is regulated, in part, by its interaction with MED1.

CHAPTER 3: GENERAL DISCUSSION

The research presented in this thesis provides evidence for MED1 as a regulator of TRα1, TRβ1, and v-ErbA nuclear retention. While previous literature explains how MED1 influences the transcriptional activity of genes controlled by TR, we expand on this by showing how the interaction is involved in dictating the subcellular distribution of TR. Our data show that in the presence of higher levels of MED1, there is a significant increase in nuclear-localized TRβ1 and v-ErbA. Similarly, in the absence of MED1, TRα1 and TRβ1 distribute more to the cytoplasm. None of these localization changes led to entire sequestration to the nucleus or cytoplasm, however. This is suggestive of either an orchestrated balance, or competition for TR-binding between exportins and MED1 to control TR's nuclear retention. Additionally, this agrees with the likelihood of several other factors, external to the described exportin-MED1 relationship, that contribute to this regulation. Also, mobility of TR variants is variably affected by EGF and T3-induced phosphorylation of MED1 by MAPK-ERK. We offered several reasons to account for inconsistencies, but ultimately, TR interacts with other intranuclear proteins that may have more resolute control over TR's mobility.

Understanding external factors affecting TR's intranuclear mobility remains important. Inducing decreases in mobility of transcription factors has been shown to be an excellent approach for revealing interactions that affect not only localization but also transcriptional activity. Using FRAP to study the

intranuclear mobility of other receptors, such as the estrogen receptor, has revealed multiple interactions that modify its interplay with DNA (Stenoien et al., 2001). Identifying these protein interactions which promote TR's residence time with DNA and decrease TR's intranuclear mobility will isolate the mechanisms controlling its nuclear retention. Ultimately, this will also help to understand where MED1 fits into the dynamics. As an example, prior studies suggest that the nuclear retention of TRβ is dependent on NCoR and dimerization with RXR (Baumann et al., 2001). As a corepressor of TR, NCoR works antagonistic to MED1, a coactivator of TR. Such relationships explain not only the requirement for transcriptional regulation by TR, but extend into the model for maintaining TR nuclear retention. Therefore, changes in TR mobility dynamics likely involves several proteins working cooperatively with each other and TR. Above all, this research provides evidence for the role of MED1 in modifying the shuttling dynamics of TR.

FUTURE DIRECTIONS

The basis of our model for a role of MED1 in TR nuclear retention arose from its capacity to affect TR gene transactivation. As we have now shown, MED1's regulation of intracellular distribution patterns extends to TRα1, TRβ1 and v-ErbA. However, MED1 has previously only been connected to altering the transcriptional output of the TRα1 isoform (Belakavadi et al., 2008; Pandey et al., 2005). Consequently, we aim to conduct a series of luciferase reporter-gene

assays to validate changes in gene transactivation of TRα1 in the presence and absence of MED1, but also probe TRβ1 and v-ErbA. From here, we will be able to compare our results against the previous studies of TRα1 and add to our characterization of MED1 affecting each of the variants. Similarly, we found that overexpressing, knocking out, and phosphorylating MED1 each had different effects. Thus, it is of interest to compare reporter gene transactivation for our targeted TR subtypes under all of these conditions.

Aside from transcriptional output, we are also interested in revealing whether MED1 facilitates regulation of TRβ1 and v-ErbA by direct interaction or indirect interaction. The more intriguing of these potential interacting partners is v-ErbA. v-ErbA contains several mutations within its LBD (Yen et al., 2001). As mentioned earlier, TRα1 and other nuclear receptors interact with MED1 directly within the AF2 region of the LBD (Fondell, 2013). An abundance of mutations within v-ErbA's LBD introduces a high possibility for failed interaction between v-ErbA with MED1. Despite this, future experiments will test for interaction between v-ErbA and MED1 using coimmunoprecipitation assays. In addition to v-ErbA, it is logical to simultaneously confirm TRβ1's interaction with MED1. A previous study characterized the interaction of TRβ1 with an alternative construct of MED1 (Zhu et al., 1997). Although the pseudo-MED1 contains the essential RBDs, it lacks several residues which may be altering the native state of the protein in undesirable ways. In summary, the narrow degree of uncertainty surrounding the interaction can easily be straightened out by running a parallel coimmunoprecipitation or pull-down assay with TRβ1.

Examining gene transactivation and binding interactions will lend support for MED1's regulation of TR isoforms, but it will also be important to integrate new findings with recent elucidation of the exportins involved with TR export. The proposal that exportins 4, 5, and/or 7 interact with NES-H12 of TRα1 and TRβ1 suggests MED1 may compete with these exportins for TR binding at this site. RNA interference was used in prior studies to exert knockdown of various exportins to reveal those that influence TR shuttling (Subramanian et al., 2015). Using a combination of this exportin-knockdown approach and TRAP220 MEFs we have the potential to find differences in TR shuttling caused explicitly from competition between MED1 and exportins for binding the NES-H12 residues.

In combination, we could couple the knock-down(-out) with two applications of shuttling assays. The first incorporates heterokaryons to examine whether overall TR shuttling is affected under desired conditions, as previously described (Bunn et al., 2001; Grespin et al., 2007). In a recent study, FRAP on binucleated cells was used to indicate any variance in the dynamics of shuttling in live cells, as well (Subramanian et al., 2015). Through these assays, we may be able to tease apart MED1's role as a coactivator for TR's intrinsic gene regulatory properties but also as an inhibitor to prevent exportins from interacting with TR helix 12, thereby elucidating a novel mechanism through which MED1 sustains TR nuclear retention. In addition, coimmunoprecipitation studies could be used to assess competition between MED1 and exportins for binding to TR, in wild-type and MED1-null cells.

An alternate hypothesis to further implicate MED1 in regulating TR's nuclear localization was initially synthesized from reports of MED1 consistently shuttling to nucleoli of cells after being phosphorylated through ERK signaling (Pandey et al., 2005). Before determining that a sufficient level of endogenous MED1 was present in HeLa cells, via western blotting, we incorporated MED1 overexpression into our nucleocytoplasmic phosphorylation assays. These pilot studies detected not only MED1 aggregates forming in the nucleoli of EGF-stimulated cells, but also largely in the cells incubated in the absence of T3 (data not shown, see Fig. 2 MED1-panel "MED1" for similar phenotype). Notably, we noticed a similar aggregate-phenotype under the same T3-absent and EGF-treated conditions with TRα1, as well (see Fig. 12A). Interestingly, another protein that displays this phenotype is p53 (Kruger and Scheer, 2010). p53 sequesters to and forms aggregates of similar frequency as TRα1 and MED1. In conjunction, Qi et al. (1997) discovered how p53 interacts with the DBD of TRα1 when T3 isn't bound, providing a possible explanation for the T3-absent phenotype we observed. Additionally, other studies have confirmed that p53 independently interacts with MED1 (Drané et al., 1997; Ito et al., 1999). Although all three proteins are paired together in transcriptional regulation and compartmental localization does not constitute interaction, these connections nonetheless point to the intriguing possibility for a novel regulatory mechanism that would ultimately add to the complexity of TR nuclear retention.

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